**Univerzita Karlova v Praze**
**3. lékařská fakulta**

# Disertační práce

Praha, 2017

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**3. lékařská fakulta**

# Disertační práce

Studium úlohy genetických faktorů v prognóze a predikci účinků chemoterapie karcinomu prsu

The role of genetic factors in the prognosis and prediction of effects of the chemotherapy of breast carcinoma

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V Praze, 17. 7. 2017

Jméno – Příjmení

Podpis

Identifikační záznam:

HLAVÁČ, Viktor. *Studium úlohy genetických faktorů v prognóze a predikci účinků chemoterapie karcinomu prsu. [The role of genetic factors in the prognosis and prediction of effects of the chemotherapy of breast carcinoma].* Praha, 2017. 55 stran, 9 příloh. Disertační práce. Univerzita Karlova, 3. lékařská fakulta, Ústav epidemiologie. Vedoucí závěrečné práce: doc. RNDr. Pavel Souček, CSc.

**Poděkování:**

Rád bych poděkoval svému školiteli doc. RNDr. Pavlu Součkovi, CSc. za vedení a pomoc při studiu i při práci na experimentech a za pomoc při psaní článků i této disertační práce. Dále bych chtěl poděkovat kolegyním v laboratoři Oddělení toxikogenomiky ve Státním zdravotním ústavu v Praze, hlavně RNDr. Radce Václavíkové, Ph.D. a Ing. Marii Ehrlichové, také kolegům a kolegyním na ostatních pracovištích, kde jsem mohl dělat část práce, za jejich pomoc i podporu. Za všechny jmenuji Ústav biochemie a experimentální onkologie, laboratoř doc. Kleibla nebo laboratoř prof. Kováře na 3. lékařské fakultě Univerzity Karlovy. Velký dík patří také všem spoluautorům, kteří jsou uvedeni v publikovaných pracích. Chtěl bych poděkovat i našim laborantům, bez kterých by experimentální práce byla mnohem těžší. Na závěr bych chtěl poděkovat své rodině za podporu a nezbytné zázemí pro vypracování této práce.

Poděkování patří též grantovým agenturám za nezbytnou finanční podporu. Tato práce byla sponsorována grantovými agenturami: GAČR, č. grantu P303/12/G163 a 13-25222J; AZV, č. grantu 15-25618A a 17-28470A; MZ ČR-RVO (SZÚ, 75010330) a částečně financována z Národního program udržitelnosti I (NPU I) č. LO1503 poskytovaného Ministerstvem školství, mládeže a tělovýchovy.

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# 1 Seznam použitých zkratek:

ABC; ATP-binding cassette transporters

AKR; aldo-keto reductases

AMPK; AMP-activated protein kinase

CBR; carbonyl reductases

CYP; cytochromes P450

DFS; disease-free survival

ER; estrogenový receptor

ERBB2/HER2; Erb-B2 receptor tyrosinkinase 2/human epidermal growth factor receptor 2

ERE; estrogen responsive element

FAC; 5-fluorouracil, anthracyklin, cyklofosfamid

FEC; 5-fluorouracil, epirubicin, cyklofosfamid

HIF-1$\alpha$; hypoxia-inducible factor-1$\alpha$

KCNAB; voltage-gated K+ channel beta subunit

MDR; multidrug resistance protein

MRP; multidrug-resistance-related protein

NGS; next-generation sequencing

OS; overall survival

P-gp; P-glykoprotein

PR; progesteronový receptor

SDR; short-chain dehydrogenases/reductases

SNP; single nucleotide polymorphism

SUR; sulphonylurea receptor

TNBC; triple-negative breast cancer

# 2 Souhrn

Karcinom prsu je nejčastějším nádorovým onemocněním žen u nás i ve světě. Jednou z překážek úspěšné léčby je mnohočetná léková resistence. Může být způsobena různými faktory, jako jsou nadměrná exprese ABC transportérů nebo nedostatečná exprese SLC transportérů, působení enzymů metabolizujících léčiva, variabilita cílů cytostatik, selhání apoptózy nebo zvýšená kapacita reparačních genů. Cílem této práce bylo hledat vztahy genů transportu a metabolismu s prognózou nebo odpovědí na léčbu pacientek s karcinomem prsu. Z hlediska preventivní medicíny je tento cíl klíčový pro naplnění potřeby jak sekundární, tak i terciární prevence nádorových onemocnění, tj. hledat způsoby, jak zvolit optimální léčbu, zabránit progresi onemocnění do obtížně léčitelných stádií a eliminovat vedlejší účinky léčby.

Expresní profil kazetových transportérů vázajících ATP (ABC, 49 genů), cytochromů P450 (CYP, 10 genů), aldoketoreduktáz (AKR, 13 genů) a karbonylreduktázy 1 byl sledován v nádorové tkáni a v okolní nenádorové kontrolní tkáni pacientek s karcinomem prsu léčených neoadjuvantní chemoterapií. Vybrané geny byly na základě rozdílné exprese mezi nádorovou a kontrolní tkání a na základě vztahů s klinickými daty stanoveny na nezávislém adjuvantně léčeném souboru pacientek a protein v nádorové tkáni byl potvrzen pomocí imunoblotingu. U vybraných kandidátních genů byla prostudována genetická variabilita.

Naprostá většina genů byla deregulována v nádorech oproti kontrolní nenádorové tkáni. Byla nalezena řada statisticky významných vztahů hladin transkriptu v nádorové tkáni s prognostickými znaky pacientek. K nejvýznamnějším patří vztah genů ABCA12, ABCA13, ABCD2, AKR1C1, AKR1C2 a CYP2W1 s odpovědí na neoadjuvantní léčbu. AKR1C2, AKR7A3, CYP3A4 a CYP2B6 souvisely s přežíváním bez příznaků onemocnění a geny ABCC1, ABCC8, AKR7A3 a CYP2B6 souvisely s expresí hormonálních receptorů v obou skupinách pacientek. V nukleotidové vazebné doméně 1 genu *ABCC1* byly nalezeny polymorfismy ovlivňující dobu bezpříznakového přežívání pacientek a v genech *ABCC8* a *ABCD2* byly cíleným exomovým sekvenováním

nalezeny dosud neznámé varianty, u kterých byl *in silico* předpovězen funkční význam.

Nově odhalené potenciální kandidátní geny a genetická variabilita v těchto genech mohou být klinicky významné. Některé z těchto genů mohou pravděpodobně ovlivňovat prognózu nebo odpověď pacientek na léčbu. Námi nalezené potenciální biomarkery prognózy a predikce účinků chemoterapie by měly být dále sledovány v navazujících studiích preklinického charakteru, kde by byl vyhodnocen jejich klinický význam a odhalen mechanismus působení.

**Klíčová slova:** *Karcinom prsu, mnohočetná léková resistence, genová exprese, biomarker, prognóza, léčebná odpověď, prevence*

## *2.1 Summary*

Breast carcinoma is the most common cancer among women in our country and worldwide. One of the obstacles to successful therapy is a multidrug resistance. It can be caused by different factors, such as overexpression of ABC transporters, or decreased expression of SLC transporters, deregulation of drug metabolizing enzymes, variability of the targets of anticancer drugs, failure of apoptosis or increased capacity of repair genes. The aim of this study was to search for associations of genes of drug transport and metabolism with the prognosis of patients or response to chemotherapy. From the view of preventive medicine, this aim constitutes an important part of both secondary and tertiary prevention of cancer, i.e., discovery of markers enabling optimal therapy selection for each patient, decreasing the risk of disease progression to advanced and resistant stage, and elimination of side effects of chemotherapy.

The expression profile of ATP-binding cassette (ABC) transporters (49 genes), cytochromes P450 (CYPs, 10 genes), aldo-keto reductases (AKRs, 13 genes) and carbonyl reductase 1 was analyzed in the tumor and adjacent non-tumor control tissues in a cohort of neoadjuvantly treated patients. Genes deregulated in tumors compared with control tissues or genes associated with clinical data were assessed in an independent set of pretreatment patients. Protein levels in tumor tissues were confirmed by immunoblotting. Genetic variability was assessed in selected candidate genes.

The vast majority of genes was deregulated in tumors compared with control tissues. A number of significant associations of intratumoral transcript levels with clinical characteristics of the patients were found. Most interestingly, transcript levels of ABCA12, ABCA13, ABCD2, AKR1C1, AKR1C2, and CYP2W1 associated with patients´ response to neoadjuvant therapy. Moreover; AKR1C2, AKR7A3, CYP3A4, and CYP2B6 associated with disease-free survival and ABCC1, ABCC8, AKR7A3, and CYP2B6 associated with the expression of hormonal receptors in both sets. Several non-coding polymorphisms in the functional nucleotide binding domain 1 of *ABCC1* gene significantly associated with disease-free survival of patients. New variants in *ABCC8* and *ABCD2* genes

were found using targeted exome sequencing and *in silico* methods predicted their functional effects.

Candidate genes which might be clinically significant were discovered. The genetic variability in some of these genes may explain prognostic or predictive roles in breast carcinoma patients. Potential biomarkers of prognosis and response of patients to chemotherapy found by this study should be further followed to confirm their clinical significance and decipher underlying mechanism of action.

**Keywords:** *Breast carcinoma, multidrug resistance, gene expression, biomarker, prognosis, chemotherapy response, prevention*

# 3 Literární úvod a přehled dané problematiky

Karcinom prsu (zhoubný novotvar prsu, C50) je nejčastějším zhoubným nádorem žen ve světě. Současně je druhou nejčastější onkologickou příčinou úmrtí ve vyspělých zemích světa (Ferlay a kol. 2014). Jeho incidence ve vyspělých zemích stále stoupá o 1–2 % ročně, zatímco mortalita pomalu klesá. Incidence a mortalita v České republice je srovnatelná s ostatními vyspělými zeměmi. V roce 2002 byl u nás zahájen screeningový program, což se projevilo vyšší incidencí, zároveň ale záchytem onemocnění v nižších stádiích. Přestože je léčba karcinomu prsu, zvláště v raných stadiích, velmi úspěšná, zůstává karcinom prsu i nadále nejčastější onkologickou příčinou úmrtí žen. V roce 2013 bylo v ČR zaznamenáno 7140 nových případů tohoto onemocnění a 1692 žen jeho následkem zemřelo. Úmrtí v důsledku karcinomu prsu tvořilo 14 % celkových úmrtí žen v důsledku zhoubných novotvarů (ÚZIS ČR 2017).

Karcinom prsu obecně zařazujeme mezi tzv. hormonálně závislé nádory. Zejména estrogeny mají karcinogenní účinky – indukují zvýšenou expresi některých růstových faktorů a také pravděpodobně onkogenů, jejichž produkty ovlivňují proliferační aktivitu buněk. Tyto popsané procesy se uplatňují u většiny tzv. sporadických forem karcinomu prsu vyskytujících se u 75–85 % nemocných. Sporadická forma nádoru prsu je pravděpodobně zapříčiněna kombinací řady faktorů genetických a environmentálních. V 10–15 % případů vzniká karcinom prsu na základě dědičných genetických změn. Od sporadické formy se liší některými epidemiologickými a klinickými příznaky. Jedinci s dědičnou mutací v predispozičních genech často onemocní v mladším věku a nádor se u nich vyskytuje většinou oboustranně a s vyšší frekvencí (Klener 2002). Tyto, tzv. hereditární, formy karcinomu prsu jsou podmíněny mutací genů BRCA-1 nebo BRCA-2 s autosomálně dominantní dědičností. Kromě těchto majoritních predispozičních genů s vysokou penetrancí je nyní studována i úloha genů s nízkou penetrancí. Závažná je zejména tzv. trojitě negativní forma karcinomu prsu (TNBC), u níž jsou nádorové buňky negativní na vyšetření exprese receptorů estrogenových (ER) i progesteronových (PR) a Erb-B2 receptoru tyrosinkinázy 2 (ERBB2/HER2). Špatná prognóza TNBC spočívá v tom, že je tato forma

hormonálně neléčitelná. TNBC tvoří asi 10–17 % všech nádorů (Perou a kol. 2000, Badve a kol. 2011).

Ve většině případů nestačí k léčbě nádorových onemocnění jen jediná léčebná metoda, ale je nutný komplexní přístup, který kombinuje chirurgickou léčbu s radioterapií a užíváním chemoterapeutik. Pro volbu chemoterapeutického režimu v první linii léčby karcinomu prsu je zásadní pozitivita ER a PR. Hormonálně pozitivní pacientky jsou obvykle léčeny látkami interagujícími s těmito receptory, tj. antiestrogeny (např. tamoxifen) a inhibitory aromatáz (Femara) nebo jejich kombinací (Jefcoate a kol. 2000; Nandi a kol. 1995). U ERBB2/HER2 pozitivních pacientek je možnost využít monoklonální protilátku trastuzumab (Herceptin). V případě hormonálně negativních pacientek je nejčastěji volen režim s kombinací anthracyklinu (tj. doxorubicin, daunorubicin, epirubicin apod.), 5-fluorouracilu a cyclofosfamidu (FAC/FEC). Pro paliativní chemoterapii metastatických nádorů prsu či u relabujících pacientek jsou pak obvykle voleny režimy založené na taxanech (paklitaxel, docetaxel). (Hořejší a kol. 1998; Klener 2002). Ke snížení nádorové masy (angl. „debulking“) před operačním výkonem v případě neoperovatelných či velkých nádorů se používá neoadjuvantní chemoterapie. Tato léčba je často založena na režimech obsahujících taxany, FAC/FEC nebo jejich kombinace. Vzhledem k tomu, že tyto režimy, zejména taxany, jsou nejen nákladné, ale také velmi toxické, je potřeba co nejpřesněji odhadnout, jaký režim na daný typ nádoru nejlépe zapůsobí. Jednou z hlavních potřeb léčby karcinomu prsu je tedy hledání nových prediktivních biomarkerů a jejich spolehlivá validace (Bonnefoi a kol. 2011; Bonnefoi a kol. 2007).

Jednou z hlavních překážek v úspěšné léčbě nádorových onemocnění je resistence nádorových buněk k léčivům. Nádorové buňky buď mohou být resistentní *per se*, nebo dojde k vytvoření resistence působením léčiva v průběhu růstu buněk. Resistence však nevzniká jen na použité léčivo, ale zpravidla na celou skupinu léčiv, které se liší strukturou i mechanismem účinku. Proto se tento jev nazývá mnohočetná léková resistence (Gottesman a kol. 2002; Gottesman a Pastan 1993). Důvody vzniku mnohočetné lékové resistence jsou nejasné, podle současné hypotézy souvisejí se změnami v mnoha metabolických a transportních

drahách. Na těchto změnách se podílejí snížené vstřebávání léčiv rozpustných ve vodě zapříčiněné sníženou expresí „solute-carrier“ (SLC) transportérů, zvýšená kapacita reparačních genů opravujících DNA poškození, snížení apoptózy, variabilita cílů cytostatik nebo zvýšený export (eflux) hydrofobních léčiv ven z buňky, za nějž jsou zodpovědné kazetové transportéry vázající ATP („ATP-binding cassette transporters“, ABC; Baguley 2010). Další příčinou selhání terapie nádorů je inaktivace cytostatika metabolismem buňky (Bray a kol. 2010, Ingelman-Sundberg a kol. 1999). Na tomto jevu se podílejí enzymy první i druhé fáze metabolismu. Mezi enzymy první fáze patří cytochromy P450 (CYP), oxidoreduktázy, karbonylreduktázy (CBR), alkoholdehydrogenázy, aldehyd-dehydrogenázy, enzymy metabolismu nukleotidů a nukleosidů apod. Enzymy druhé fáze zahrnují glutathion-S-transferázy, N-acethyltransferázy, sulfo-transferázy, UDP-glukuronosyltransferázy apod. (Souček 2008).

## *3.1 ABC transportéry*

ABC transportéry tvoří velkou nadrodinu genů, které se funkčně a strukturně dělí do mnoha podrodin. U rostlin známe více než stovku ABC transportérů. U člověka je známo 48 funkčních ABC transportérů a jeden pseudogen (ABCC13). Lidské ABC transportéry se dělí do sedmi rodin, A až G. Jejich funkcí je transport látek přes extracelulární a intracelulární membrány. Transport zahrnuje širokou škálu substrátů, jako jsou produkty látkové výměny, lipidy, steroly a cizorodé látky neboli xenobiotika (Dean a kol. 2001). ABC jsou organizované jako úplné transportéry obsahující dvě N-terminální transmembránové a dvě C-terminální nukleotidové vazebné domény nebo jako poloviční transportéry, obsahující pouze jednu od každé z těchto domén; které, aby byly plně funkční, musejí vytvářet homodimery, popř. heterodimery (Higgins 1992). Mnohočetná léková resistence způsobená efluxem cytostatika z nádorových buněk bývá nejčastěji spojena s nadměrnou expresí, především díky indukci (zvýšení exprese působením cytostatika), jednoho nebo více ABC transportérů (Leonessa a Clarke 2003; Szakács a kol. 2006). Nejvíce studovaným ABC transportérem je gen *ABCB1*. Jeho produktem je protein mnohočetné lékové resistence („multidrug resistance protein“, MDR) 1, nazývaný též P-glykoprotein

(P-gp; Wolf a kol. 2011; Zhang a kol. 2001; Ueda a kol. 1986). Nejvíce je exprimován v játrech, ledvinách, střevech, mozku, děloze, varlatech a placentě (Hugger a kol. 2002; Kuwano a kol. 2003). Zvýšená exprese *ABCB1*/P-gp byla v některých pracích spojena se špatnou odpovědí na léčbu pacientů s karcinomem prsu (Atalay a kol. 2008; Burger a kol. 2003), zatímco jiní autoři žádný vztah *ABCB1*/P-gp k prognóze karcinomu prsu nenalezli (Larkin a kol. 2004; Moureau-Zabotto a kol. 2006; Vaclavikova a kol. 2008). Dalším studovaným ABC transportérem je *ABCC1*. Jeho produktem je „multidrug-resistance-related protein" (MRP) 1 zodpovědný za mnohočetnou lékovou resistenci různých solidních nádorů (Cole a kol. 1991). V literatuře byla popsána silná korelace mezi hladinou exprese ABCC1 a sníženou dobou do relapsu i celkového přežívání („overall survival", OS; Rudas a kol. 2003). Oproti tomu v jiných studiích vztah mezi ABCC1 a prognózou karcinomu prsu nalezen nebyl (Szakács a kol. 2006). Mezi třetí nejčastěji studovaný ABC transportér patří gen *ABCG2*, jehož produktem je „breast cancer resistence protein" (BCRP; Nakanishi a kol. 2003; Natarajan a kol. 2012; Doyle a kol. 1998).

ABC proteiny z rodiny A (ABC1) jsou zodpovědné především za transport sterolů a lipidů. ABC proteiny z rodiny B jsou proteiny mnohočetné lékové resistence a transportéry spojené se zpracováním antigenů („transporter associated with antigen processing", MDR/TAP), jsou zodpovědné za transport léčiv (P-gp), hemu a žlučových kyselin. Rodina C (MRP) obsahuje transportéry zodpovědné za mnohočetnou lékovou resistenci. Jejich substráty jsou aniontové sloučeniny, produkty II. fáze metabolismu, steroidní hormony apod. Vyskytují se spolu s P-gp v hematoencefalické bariéře, kde fungují jako pumpy zajišťující neprostupnost bariéry pro tyto látky. Dále obsahuje členy kódující iontové kanály související s cystickou fibrózou („cystic fibrosis transmembrane conductance regulator", CFTR) a diabetes mellitus („sulphonylurea receptor", SUR). Rodina D, „adrenoleukodystrophy" (ALD) je zodpovědná za transport lipidů z peroxisomů do cytoplasmy a je spojená s nemocí adrenoleukodystrofií. Rodina E („organic anion binding protein", OABP) obsahuje jediný transportér ABCE1, který váže organické anionty a není v pravém slova smyslu transportér, ale spíše inhibitor ribonukleáz, respektive faktor účastnící se iniciace translace. Rodina F obsahuje

tři členy, jež jsou odvozeny od ABC transportérů, ale postrádají transmembránové domény. Transportéry z rodiny G („white“) jsou zodpovědné za transport různých produktů látkové výměny, xenobiotik, včetně cytostatik, a žlučových kyselin (Dean a kol. 2001; Allikmets a kol. 1996; humanabc.4t.com).

Jedním z nejdůležitějších výsledků v publikaci (Hlaváč a kol. 2013), která je součástí této komentované disertační práce, je vztah hladin transkriptu genů ABCC1 a ABCC8 s prognózou pacientek a genu ABCD2 s odpovědí na léčbu.

### 3.1.1 ABCC1 (MRP1)

Jedním z nejvíce studovaných ABC transportérů je *ABCC*1/MRP1. Vyskytuje se téměř ve všech tkáních. Vysoké hladiny se nacházejí v plicích, varlatech, ledvinách, kosterním a srdečním svalu a v placentě. Je negativním prognostickým markerem řady různých karcinomů: *in vitro* karcinomu plic a prostaty (Cole a kol. 1992; Zalcberg a kol., 2000), *in vivo* karcinomu prsu (Rudas a kol. 2003; Yamada a kol. 2013) a jiných (Kunická a Souček 2014). Skládá se ze dvou nukleotidových vazebných domén se dvěma konzervovanými motivy Walker A a Walker B a ze třech transmembránových domén, skládajících se celkem ze 17 transmembránových helixů (Cole a kol. 2014). Sloučeniny exportované z buněk jsou většinou anionty, často konjugované s glutathionem, glukuronidem nebo sulfátem, anebo jsou transportovány nekonjugované s volným glutathionem (Borst a kol. 1999). Většina z genetických změn v tomto genu se skládá z jednonukleotidových polymorfismů („single nucleotide polymorphism“, SNP), méně časté jsou repetice, inzerce a delece. K dnešnímu dni bylo identifikováno 2352 SNP. Asi 1 % z těchto polymorfismů představují nesynonymní záměny, které mění uspořádání aminokyselin v proteinu (Kunická a Souček 2014).

### 3.1.2 ABCC8, sulfonylmočovinový receptor 1 (SUR1)

Na rozdíl od většiny ABC transportérů ABCC8 nefunguje jako transportér léčiv, ale spolu s proteinem Kir6.2 („inwardly rectifying $K^+$ channel“) tvoří $K^+$ kanál řízený molekulou ATP. Tento kanál se působením ATP (tedy při zvýšeném metabolismu v přítomnosti glukózy) uzavírá a způsobuje depolarizaci membrán β-

buněk pankreatu s následnou sekrecí insulinu. Mutace v genu *ABCC8* jsou spojovány s různými formami glykemických poruch, jako je familiární hyperinsulinemická hypoglycemie 1. typu nebo neonatální diabetes mellitus. Sulfonylmočovina stimuluje sekreci insulinu z β-buněk pankreatu přímo, proto je používána v léčbě diabetu 2. typu i neonatální diabetes (Aittoniemi a kol. 2009; de Wet a kol. 2010). Vztah genetických variant *ABCC8* ke vzniku diabetu mellitu popsal v review Haghverdizadeh a kol. (2014). O vztahu fenotypu ABCC8 k progresi nádorů je dostupných informací málo, ale v současnosti byla popsána souvislost mezi nadměrnou expresí ABCC8 a prostupností hematoencefalické bariéry ve zvířecím modelu mozkových metastáz, přičemž inhibice ABCC8 glyburidem zvýšila protinádorový účinek (Thompson a kol. 2013).

### 3.1.3 ABCD2, adrenoleukodystrofický protein (ALDRP)

ABCD2 kóduje produkt spojený s adrenoleukodystrofií („adrenoleukodystrophy-related protein“, ALDRP). Tento protein je velmi podobný ABCD1, sdílí s ním 63 % homologie a částečnou substrátovou specifitu. Substráty obou enzymů jsou nasycené a mononenasycené mastné kyseliny transportované přes membránu liposomů, ABCD2 má však substrátovou specifitu posunutou ke kratším (C20: 0 a C22-CoA: 0-CoA) a polynenasyceným mastným kyselinám (C22: 6-CoA a C24: 6-CoA). Existují údaje o tom, že se tento gen podílí na diferenciaci buněk a vývoji nádorového onemocnění. Je spojen s obezitou, jaterní steatózou a insulinovou resistencí (Liu a kol. 2012; Barbet a kol. 2012). Zajímavé je, že nedávno byla *in vitro* prokázána přímá regulace ABCD2 β-kateninem a transkripčním faktorem 4 (TCF4; Park a kol. 2013). Tyto transkripční faktory jsou regulátory Wnt signalizace související se vznikem nádorů tlustého střeva a pankreatu (Bienz a Clevers 2000; Jones a kol. 2008).

## *3.2 Cytochromy P450*

Biotransformační reakce se rozdělují na reakce I. fáze (funkční) a reakce II. fáze (konjugační; Daly a kol. 1993). V I. fázi dochází k zabudování hydrofilní funkční skupiny (OH) do molekuly substrátu. Ve II. fázi je tato skupina použita pro konjugaci s glutathionem, sulfátem, kyselinou glukuronovou, glukosou nebo

cysteinem a dochází k přeměně na polárnější látku hydrofilní povahy (Vineis a kol. 1999). Biotransformační enzymy se však, mimo těchto prospěšných detoxikačních reakcí, účastní také aktivace prokarcinogenů na mutageny a karcinogeny. Tento obecně negativní děj se může stát i prospěšným v situaci, kdy aktivací cytostatik dojde k poškození biologických makromolekul (DNA, RNA, proteiny, lipidy) v nádorových buňkách během chemoterapie (Stiborová a kol. 1999).

CYP monooxygenázy se účastní první fáze biotransformace, která typicky zahrnuje oxidaci substrátů: cizorodých látek, produktů látkové výměny a endogenních látek (např. steroidních hormonů, vitamínů apod.). Rozdělují se funkčně a strukturně do rodin podle aminokyselinové sekvence, fylogenetických kritérií a genové organizace (Nelson a kol. 1996). Nejvíce zastoupené isoformy v játrech jsou CYP3A, 2C, 1A1, 2E1, dále v malé míře CYP2D6 a více než třetinu tvoří další CYP, zatímco největší podíl na metabolismu substrátů v játrech má CYP3A (více než 50 %), daleko méně CYP2D6, 2C, 2E1 a 1A2 (Shimada a kol. 1994). Asi dvacet enzymů z 57 známých lidských CYP se účastní metabolismu prokarcinogenů a léčiv. Většině z nich chybí důležité funkční polymorfismy, avšak *CYP2A6, 2B6, 2C9, 2C19* a *2D6* jsou vysoce polymorfní, což může mít účinek na jejich expresi a výslednou aktivitu (Johansson a Ingelman-Sundberg 2011).

V naší druhé publikované práci (Hlaváč a kol. 2014) byl nalezen významný vztah exprese CYP2B6 s prognózou pacientek a vztah genu CYP3A4 s přežíváním bez příznaků onemocnění („disease-free survival“, DFS) pacientek po chemoterapii.

### 3.2.1 CYP2B6

CYP2B6 katalyzuje oxidaci širokého spektra substrátů s preferencí lipofilních středně velkých neutrálních nebo slabě zásaditých sloučenin. V porovnání s ostatními podrodinami CYP je poměrně málo konzervován mezi různými savčími druhy (Wilderman a Halpert 2012). CYP2B6 se účastní také aktivace cyklofosfamidu v játrech, avšak jeho vliv na odpověď na léčbu onkologických pacientů nebyl dosud prokázán (Rodriguez-Antona a kol. 2010).

Byla pozorována nadměrná exprese mRNA genu CYP2B6 v nádorech prsu pacientek s expresí ER v porovnání s nádory pacientek bez exprese ER (Bieche a kol. 2004; Tozlu a kol. 2006). V nedávné době se prokázalo, že exprese CYP2B6 je regulována ER přímou vazbou na estrogen responsivní element („estrogen responsive element“, ERE) vyskytující se v promotoru genu *CYP2B6* (Lo a kol. 2010).

### 3.2.2 CYP3A4

Jedním z nejvíce zastoupených enzymů v játrech je rodina CYP3A (Shimada a kol. 1994). CYP3A4 má velkou podobnost i substrátové překrytí s CYP3A5 (Daly a kol. 2006; de Wildt a kol. 1999). Spolu s CYP2C9, CYP2C19 a CYP3A5 se účastní aktivace cyklofosfamidu v játrech (Roy a kol. 1999; Chang a kol. 1993) CYP3A4 a vedle něj též CYP2C8 a CYP3A5 jsou hlavními enzymy metabolizujícími taxany (Rahman a kol. 1994; Shou a kol. 1998). Jednoznačně byla prokázána exprese CYP3A4 v prsní žláze na úrovni mRNA (Iscan a kol. 2001; Modugno a kol. 2003). Vysoké hladiny CYP3A4 na úrovni transkriptu (Miyoshi a kol. 2002) nebo proteinu (Miyoshi a kol. 2005) predikují špatnou odpověď na docetaxel u pacientek s karcinomem prsu. V jiné práci byla silná exprese proteinů CYP2S1 a CYP3A4 spojena s kratší dobou přežití pacientek s karcinomem prsu (Murray a kol. 2010).

## *3.3 Aldoketoreduktázy*

Aldoketoreduktázy (AKR) a draslíkové kanály řízené napětím (KCNAB) se účastní redoxních transformací širokého spektra xenobiotik obsahujících karbonylovou skupinu. Příkladem může být transformace adriamycinu (obchodní název doxorubicinu) na jeho neaktivní metabolit adriamycinol (Barski a kol. 2008; Wermuth a kol. 1986; Thorn a kol., 2011). Savčí aldoketoreduktázy jsou rozděleny do 3 rodin: AKR1, KCNAB a AKR7; celkem je identifikováno třináct AKR proteinů: AKR1A1 (aldehydreduktázy), AKR1B1 a AKR1B10 (aldózoreduktázy), AKR1C1, AKR1C2, AKR1C3 a AKR1C4 (hydroxysteroid-dehydrogenázy), AKR1D1 (Δ4-3-ketosteroid-5-β-reduktáza), KCNAB1,

KCNAB2 a KCNAB3 (draslíkové kanály řízené napětím) a AKR7A2 a AKR7A3 (aflatoxinreduktázy; Barski a kol., 2008).

V naší studii (Hlaváč a kol. 2014) byl nalezen vztah mezi hladinami transkriptů genů AKR1C1 a AKR1C2 a odpovědí pacientek na léčbu. Pacientky odpovídající na léčbu měly významně vyšší hladiny AKR1C1 a AKR1C2 v nádorech než pacientky bez odpovědi. Byl nalezen též vztah mezi vyššími hladinami transkriptů AKR1C2 a AKR7A3 a delším DFS.

### 3.3.1 AKR1C1/AKR1C2

Tyto geny, které se v průběhu evoluce vyvinuly ze společného genu, mají vysokou homologii, 86 % mezi sebou i mezi celou podrodinou C1 – C4, vyskytující se na chromosomu 10p15-p14 (Barski a kol. 2008). Katalyzují přeměnu aldehydů a ketonů na alkoholy za pomoci kofaktorů NADH a/nebo NADPH. Mají překrývající se, ač poněkud rozdílnou, substrátovou specifitu. AKR1C1 hraje roli v inaktivaci progesteronu na 20α-hydroxyprogesteron. Porucha genu AKR1C1 je spojena s nemocemi, např. vývojovou dysplasií kyčle a ischemickou kostní chorobou, rovněž může hrát úlohu v transportu žluči (GeneCards, http://genecards.org). AKR1C2 je nejvíce exprimován v prostatě a mléčné žláze a je zodpovědný za transport žluči v hepatocytech (Barski a kol. 2008). Mezi nemoci spojené s AKR1C2 patří pseudohermafroditismus („46XY sex reversal 8“, OMIM: 614279). Enzym AKR1C2 má též slabou 3α-hydroxysteroiddehydrogenázovou aktivitu, metabolizuje progesteron na inaktivní 3α-hydroxyprogesteron. Spolu s 5α/5β-steroidreduktázou katalyzuje inaktivaci potentního adrogenu 5α-dihydrotestosteronu na 3α-diol (GeneCards).

### 3.3.2 AKR7A3

Dalším kandidátním genem je AKR7A3. Jedná se o aflatoxin reduktázu, metabolizující aflatoxin B1-dialdehyd na netoxický alkohol. Proteiny AKR7A3 fungují jako dimery (Barski a kol. 2008; Guengerich a kol. 2001). O tomto genu a vztahu k nádorům není v literatuře mnoho známo. Rezaul a kol. (2010) pozoroval vyšší expresi proteinu AKR7A3 ve vzorcích pacientek s ER pozitivním

karcinomem prsu. Existuje i spojení AKR7A3 s adenokarcinomem pankreatu (GeneCards) a hepatocelulárním karcinomem (Albrethsen a kol. 2011).

## *3.4 Karbonylreduktázy*

Karbonylreduktázy patří do skupiny dehydrogenáz/reduktáz s krátkým řetězcem (SDR). Mezi její zástupce patří geny CBR1 (SDR21C1), CBR3 (SDR21C2) a CBR4 (SDR45C1). Podobně jako aldoketoreduktázy se účastní redoxních přeměn xenobiotik obsahujících karbonylovou skupinu. CBR1 a CBR3 se účastní metabolismu doxorubicinu a daunorubicinu na jejich neaktivní kardiotoxické metabolity (doxorubicinol a daunorubicinol) a ovlivňují odpověď pacientů na protinádorovou terapii. Zejména hladiny proteinu a nesynonymní SNP byly spojeny s chemoresistencí a kardiotoxicitou (Hofman a kol. 2015; Malátková a kol. 2010).

V naší studii (Hlaváč a kol. 2014) byl objeven vztah hladin transkriptu genu CBR1 s DFS pacientek po adjuvantní chemoterapii a s histologickým stupněm („grade“) nádoru. Pacientky s nádory grade 3 měly významně vyšší hladiny než pacientky s grade 1 nebo 2.

### 3.4.1 CBR1

Tato NADPH-dependentní karbonylreduktáza metabolizuje široké spektrum substrátů obsahující karbonylovou skupinu, např. chinony, prostaglandiny, menadion a různá xenobiotika (např. doxorubicin). Gen CBR1 je součástí metabolické dráhy kyseliny arachidonové, prekurzorové molekuly prostaglandinu E2 (Shen et al. 2014). CBR1 přeměňuje prostaglandin E2 na prostaglandin F2-α. Váže též glutathion a redukuje S-nitrosoglutathion. S tímto genem je spojována preeklampsie/eklampsie 5 (onemocnění s autosomálně dominantní dědičností; GeneCards). V současné literatuře existuje dostatek důkazů o působení CBR1 na účinnost léčby karcinomu prsu s použitím doxorubicinu (Lal a kol. 2008; Jo a kol. 2017).

# 4 Cíle Práce

Hlavním cílem práce bylo hledat rozdíly v expresi genů metabolismu a transportu cytostatik mezi nádorovými tkáněmi pacientek dobře odpovídajících a tkáněmi pacientek špatně odpovídajících na standardní chemoterapii karcinomu prsu a také mezi pacientkami lišícími se v prognostických a klinických znacích. V pilotní studii, která probíhala na 68 pacientkách léčených neoadjuvantní chemoterapií, byly identifikovány potenciální biomarkery, které by mohly ovlivňovat prognózu nebo výsledek léčby pacientek s karcinomem prsu.

Dalším cílem projektu bylo tyto kandidátní geny validovat na větší nezávislé skupině pacientek a odhalit mechanismus resistence, jednak prostudováním korelace genové exprese, vyjádřené hladinou mRNA, s hladinou proteinu imunochemicky pomocí imunoblotingu, a dále zkoumáním, zda je resistence vrozená (tj. způsobená genetickými polymorfismy) nebo indukovaná přítomností cytostatika.

## *4.1 Hypotézy*

Pracovní hypotéza vychází z předpokladu, že existují genetické biomarkery, které korelují s výsledkem chemoterapie karcinomu prsu. Existence těchto markerů byla naznačena řadou studií za pomoci modelových buněčných linií a transgenních zvířat. V předchozích studiích na pracovišti školitele byla identifikována celá řada těchto genetických markerů, které se významně liší mezi tkáněmi pacientek s karcinomem prsu, korelují s jejich prognózou i s výsledkem chemoterapie (Václavíková a kol. 2007a, 2007b, 2008, 2012; Hubáčková a kol. 2009, 2012; Brynychová a kol. 2013). Očekávaným výsledkem byla identifikace genů, jejichž produkty přímo způsobují snížení účinku chemoterapie karcinomu prsu či s tímto snížením pouze korelují, tj. u nichž mechanismus působení nebude ihned zřejmý. Tato práce měla přispět k prevenci onemocnění karcinomu prsu definováním omezeného počtu kandidátních genů z funkčně významných skupin a nalezením mechanismu resistence za účelem volby vhodné metody predikce. Sledování variability DNA (např. polymorfismů v lymfocytech z krve) je

významně jednodušší a levnější než analýza genových expresí či používání nákladných protilátek pro imunohistochemii.

# 5 Materiál, pacienti a metody

Materiál použitý v experimentech je popsaný v pracích, které jsou součástí disertační práce (Souček a kol., 2015; Kunická a kol. 2014; Hlaváč a kol., 2014; Hlaváč a kol., 2013).

Sběr vzorků, hodnocení kvality a kvantifikace RNA, DNA, proteinu a příprava a preamplifikace cDNA jsou popsány jinde (Hlaváč a kol., 2013; Vaclavikova a kol., 2012; Hubackova a kol. 2009; Souček a kol. 2005). Všichni pacienti, jejichž vzorky a data byly studovány, potvrdili podpisem Informovaného souhlasu pacienta s účastí ve studii svoji ochotu účastnit se studie. Studie byla schválena etickou komisí Státního zdravotního ústavu v Praze a spolupracujících poskytovatelů zdravotní péče (FN Motol, Medicon a.s. a Nemocnice Atlas ve Zlíně).

# 6 Výsledky

Jedním z nejdůležitějších výsledků bylo nalezení vztahu hladin transkriptu s odpovědí pacientek na neoadjuvantní léčbu hodnocenou metodou podle RECIST („Response evaluation criteria in solid tumors“; Therasse a kol. 2000). Pacientky s částečnou odpovědí na léčbu měly v nádorové tkáni významně vyšší hladiny transkriptů genů ABCD2, ABCA13, AKR1C1, AKR1C2 a CYP2W1 než pacientky se stabilním nebo progresivním onemocněním. Naopak pacientky s onemocněním ve stádiu progrese nebo pacientky se stabilním onemocněním měly významně vyšší hladinu transkriptu genu ABCA12 v kontrolní nenádorové tkáni než pacientky odpovídající na léčbu dobře (Obrázek 1). Dále byly nalezeny vztahy mezi hladinami transkriptu genů ABCC1, ABCC8 a CYP2B6 a expresí ER, PR i stupněm vyzrávání neboli grade nádorů pacientek. Hladiny transkriptů genů ABCD2, AKR1C2 a CYP3A4 významně asociovaly s DFS pacientek po neoadjuvantní chemoterapii (Obrázky 2 a 3). Hladiny genů AKR7A3, CYP2B6 a CBR1 asociovaly s DFS pacientek, jež neměly indikovánu žádnou předoperační léčbu a jež byly posléze léčeny buďto jakoukoli adjuvantní terapií (AKR7A3, CYP2B6; n = 50 pacientek) nebo pouze adjuvantní chemoterapií (AKR7A3, CBR1; n = 25; Obrázky 4 a 5). V případě pacientek léčených adjuvantní terapií (validační skupina, n = 88) nebyl nalezen signifikantní vztah hladin transkriptu ABC transportérů s dobou přežívání bez progrese onemocnění (Obrázek 6).

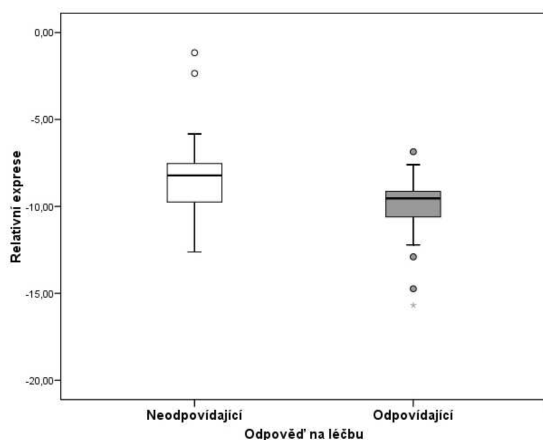
Na základě těchto výsledků byla ověřena exprese kandidátních genů v nádorových tkáních též na úrovni proteinu. Imunoblotingem byly stanoveny hladiny proteinů v nádorových tkáních pacientek u těchto kandidátních biomarkerů: ABCC1, ABCC8, ABCD2, ABCA12, AKR1C1, AKR1C2, AKR7A3, CBR1 a CYP3A4 (Obrázky 7 a 8). Exprese proteinů CYP2B6 a CYP2S1 nebyla v nádorech detekována, ačkoli protilátky rozeznávaly specificky kontrolní proteiny.

Dále byla sledována genetická variabilita kandidátních biomarkerů. V genech *ABCC1*, *ABCD2* a *ABCC8* byl *in silico* sledován funkční význam polymorfismů i nově nalezených variant.

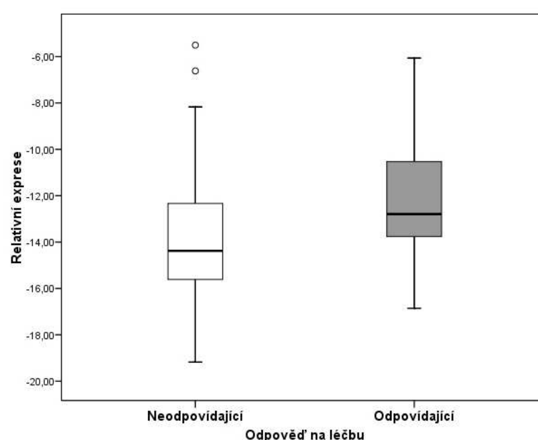
Nekódující polymorfismy v nukleotidové vazebné doméně 1 genu *ABCC1* byly analyzovány pomocí přímého sekvenování. Nádory pacientek s divokým („wild type“) genotypem v polymorfismech rs35623 nebo rs35628 měly signifikantně nižší hladiny transkriptu ABCC1 než nositelky minoritní alely. Dále se hladiny transkriptu statisticky významně zvyšovaly v pořadí: CT-GT>CC-GT>CC-GG v predikovaném diplotypu rs35626-rs4148351 složeném ze dvou polymorfismů. Chemoterapeuticky léčené nosičky alely T polymorfismu rs4148353 měly signifikantně delší DFS než pacientky s genotypem GG. Naopak hormonálně léčené pacientky s genotypem AA v rs35628 měly signifikantně delší DFS než nosičky alely G (Obrázek 9).

Geny *ABCD2* a *ABCC8* byly sekvenovány pomocí paralelního sekvenování, nazývaného častěji sekvenování nové generace („next-generation sequencing“, NGS). Bylo nalezeno množství genetických variant v obou genech. Z celkem 113 nalezených variant bylo 83 v nekódujících úsecích, 13 variant představovaly synonymní záměny (změny v kódující sekvenci neměnící pořadí aminokyselin), 10 posunů čtecího rámce a 7 variant tvořily nesynonymní záměny aminokyselin (rozložení variant viz Obrázek 10, A). Predikčními algoritmy byly označeny 3 varianty jako potenciálně škodlivé pro funkci proteinu a 3 varianty v regulačních oblastech byly predikovány jako mutace ovlivňující vazbu transkripčních faktorů. Jedna varianta v genu *ABCC8* se nalézá v nukleotidové vazebné doméně, tedy má potenciální dopad na funkci proteinu (Obrázek 10, B).

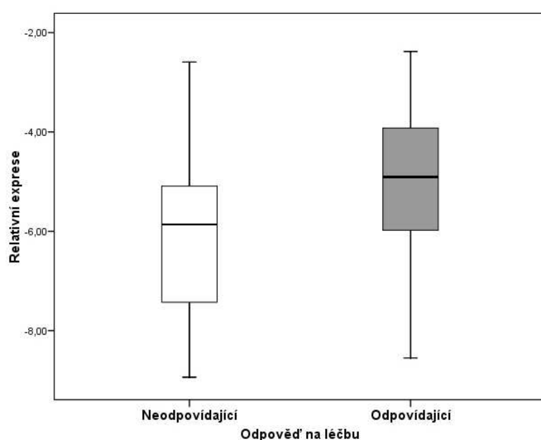
ABCA12 (P = 0,048)



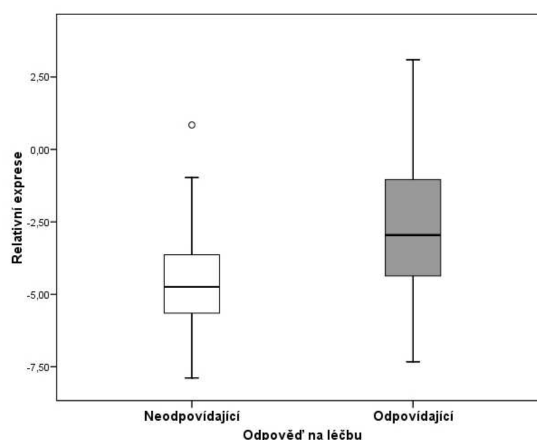

ABCA13 (P = 0,048)



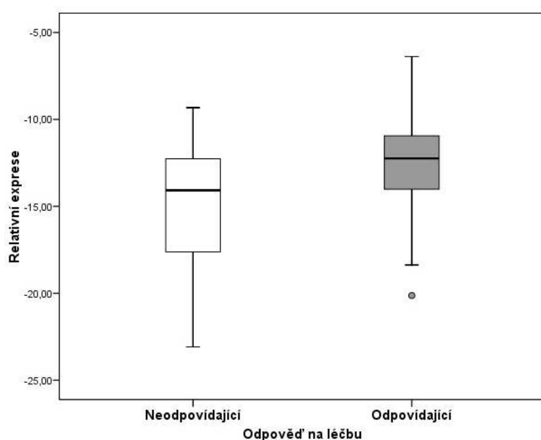

ABCD2 (P = 0,024)



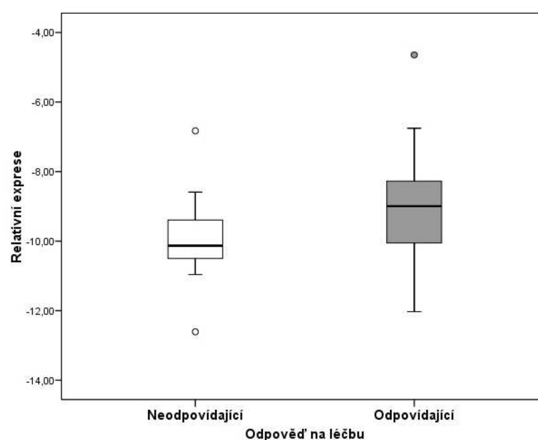

AKR1C1 (P = 0,003)




AKR1C2 (P = 0,016)

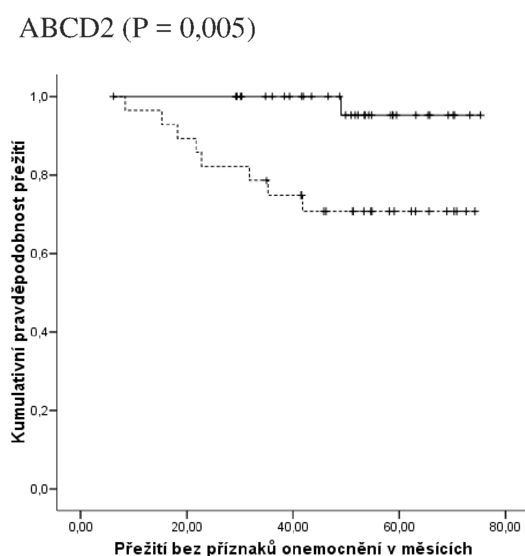



CYP2W1 (P = 0,025)




**Obrázek 1: Významné vztahy mezi hladinami transkriptu a odpovědí pacientek na neoadjuvantní léčbu.** Hladiny transkriptu v nádorové tkáni (kromě genu ABCA12, u kterého byla sledována exprese v okolní nenádorové tkáni) na ose y jsou vyjádřené pomocí hodnot ΔCt normalizovaných na referenční geny (ΔCt = průměr $Ct_{\text{referenční geny}}$ – $Ct_{\text{sledovaný gen}}$). Na obrázku jsou vyznačeny názvy genů a hladiny významnosti rozdílu mezi skupinami.

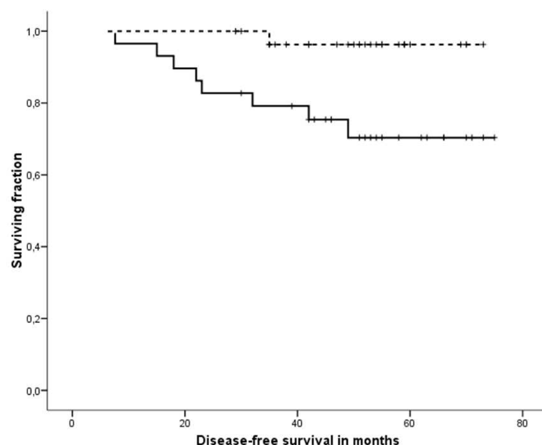
Pozn.: Ct = „cycle threshold", cyklus, ve kterém amplifikační křivka překročí prahovou hodnotu



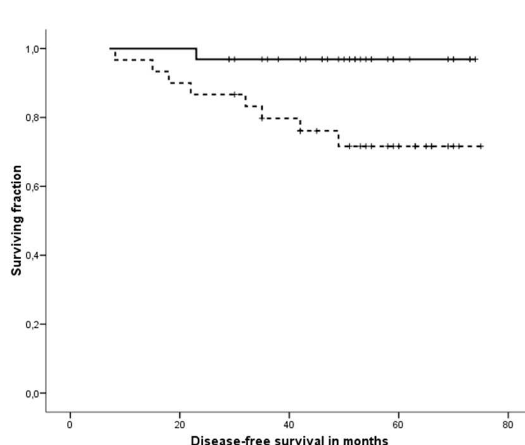

**Obrázek 2: Vztah genové exprese ABCD2 k bezpříznakovému přežití neoadjuvantně léčených pacientek.** Kaplan-Meierovy křivky přežívání byly vyneseny pro pacientky (n = 63) rozdělené do dvou skupin na základě mediánu hladin transkriptů v nádorové tkáni. Čárkovaná čára reprezentuje skupinu s nižšími hladinami transkriptu (n = 29; DFS = 59,76 ± 4,38 měsíců) a plná čára reprezentuje skupinu s vyššími hladinami než medián (n = 34; DFS = 73,82 ± 1,16 měsíců). Rozdíl mezi skupinami byl porovnán pomocí log-rank testu. Na obrázku je vyznačen název genu a hladina významnosti rozdílu mezi skupinami. Coxův proporcionální hazardní model na základě multiparametrického testování (které bere do úvahy expresi ER, grade, zasažení uzlin metastázami a velikost nádoru) potvrdil tento vztah (P = 0,033; HR = 9,60; 95% IS = 1,20 – 76,81).

Pozn.: ER = estrogenový receptor; HR = poměr rizik, „hazard ratio"; IS = 95% interval spolehlivosti; DFS = přežití bez příznaků onemocnění, „disease-free survival"

AKR1C2 (P = 0,015)

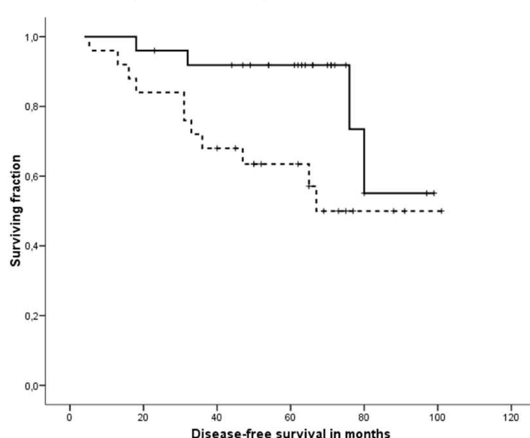



CYP3A4 (P = 0,007)

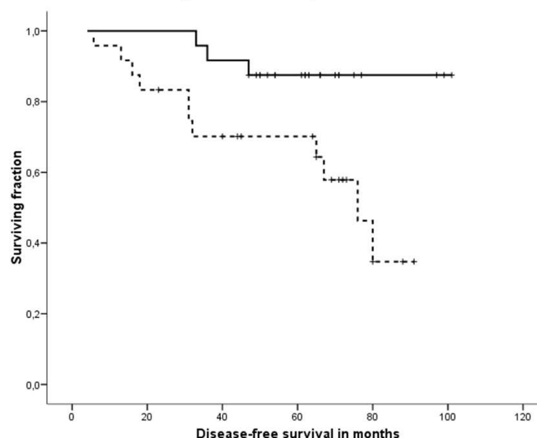



**Obrázek 3: Vztahy mezi hladinou genové exprese a bezpříznakovým přežitím neoadjuvantně léčených pacientek.** Kaplan-Meierovy křivky přežívání byly vyneseny pro pacientky (n = 65) rozdělené do dvou skupin na základě mediánu hladin transkriptů v nádorové tkáni. Čárkované čáry reprezentují skupinu s nižšími hladinami transkriptu a plné čáry reprezentují skupinu s vyššími hladinami než medián. Rozdíly mezi skupinami byly porovnány pomocí log-rank testu. Jsou vyznačeny názvy genů a hladiny významnosti rozdílů mezi skupinami.

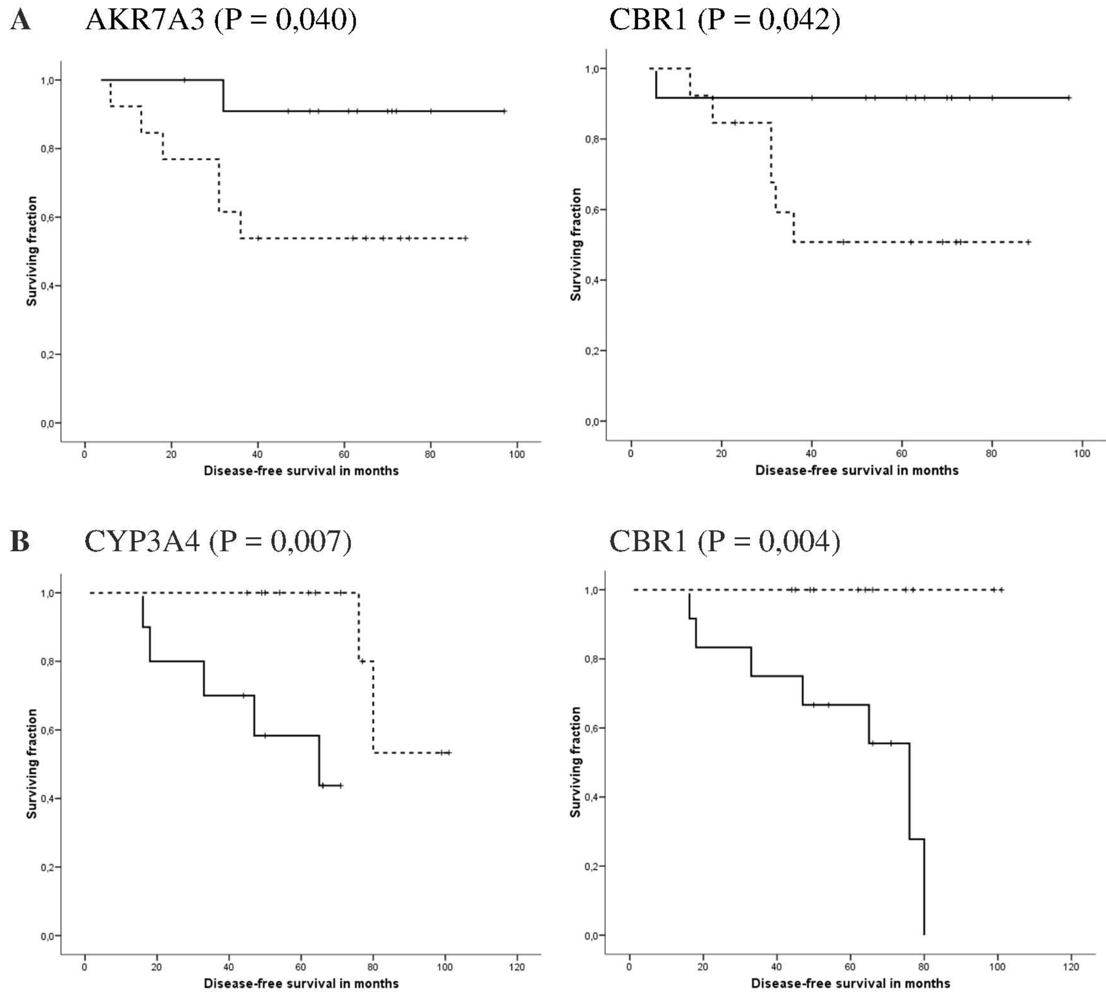
AKR7A3 (P = 0,032)



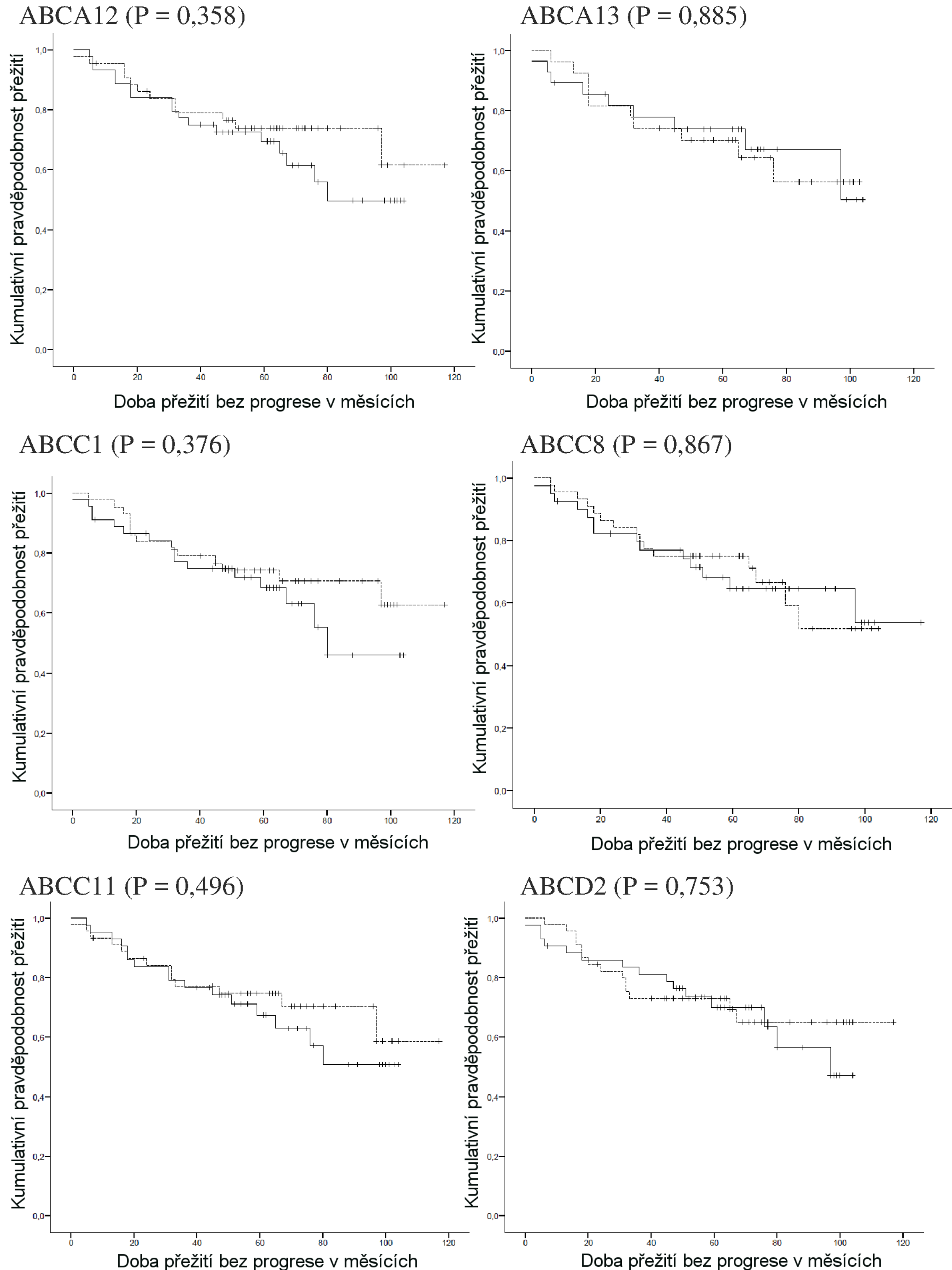

CYP2B6 (P = 0,019)




**Obrázek 4: Vztahy mezi hladinou genové exprese a bezpříznakovým přežitím adjuvantně léčených pacientek.** Kaplan-Meierovy křivky přežívání byly vyneseny pro pacientky (n = 50) rozdělené do dvou skupin na základě mediánu hladin transkriptů v nádorové tkáni. Čárkované čáry reprezentují skupinu s nižšími hladinami transkriptu a plné čáry reprezentují skupinu s vyššími hladinami než medián. Rozdíly mezi skupinami byly porovnány pomocí log-rank testu. Jsou vyznačeny názvy genů a hladiny významnosti pro rozdíly mezi skupinami.



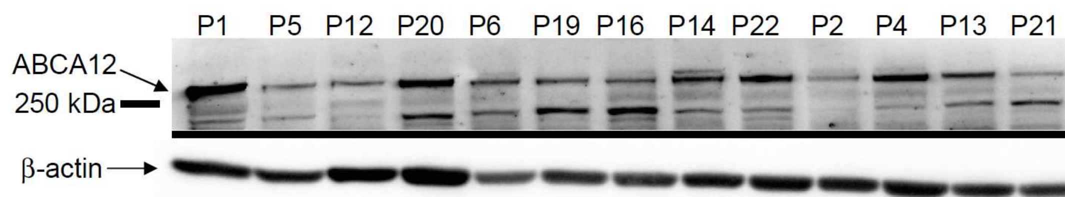

**Obrázek 5: Vztahy mezi hladinou genové exprese a bezpříznakovým přežitím neoadjuvantně léčených pacientek rozdělených podle typu terapie.** Kaplan-Meierovy křivky přežívání byly vyneseny pro pacientky léčené chemoterapií (**A,** n = 25) a hormonální terapií (**B**, n = 23) rozdělené do dvou skupin na základě mediánu hladin transkriptů v nádorové tkáni. Čárkované čáry reprezentují skupinu s nižšími hladinami transkriptu a plné čáry reprezentují skupinu s vyššími hladinami než medián. Rozdíly mezi skupinami byly porovnány pomocí log-rank testu. Jsou vyznačeny názvy genů a hladiny významnosti rozdílů mezi skupinami.



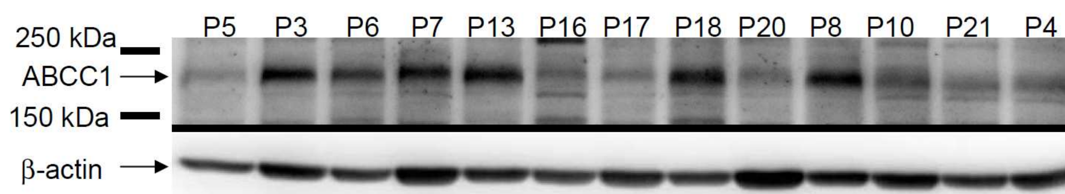

**Obrázek 6: Grafy přežití pacientek bez progrese pro ABC transportéry analyzované v ověřovací studii.** Pacientky ze skupiny léčené adjuvantní terapií (n = 88) byly rozděleny do skupin podle mediánu hladin transkripce v nádorech. Kaplan-Meierovy grafy jsou vykresleny pro obě skupiny (čárkovaná čára značí skupinu s vyšší hladinou transkriptu a plná čára značí skupinu s nižší hladinou transkriptu než

medián). Rozdíly mezi skupinami jsou porovnány pomocí log-rank testu. Jsou vyznačeny názvy genů a hladiny významnosti rozdílů mezi skupinami.

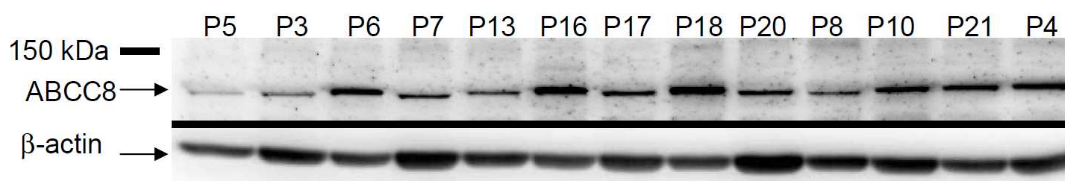
**A – ABCA12 (predicted MW 257 kDa)**



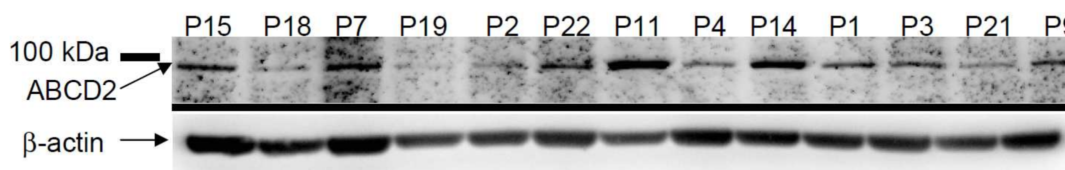

**B – ABCC1 (predicted MW 190 kDa)**



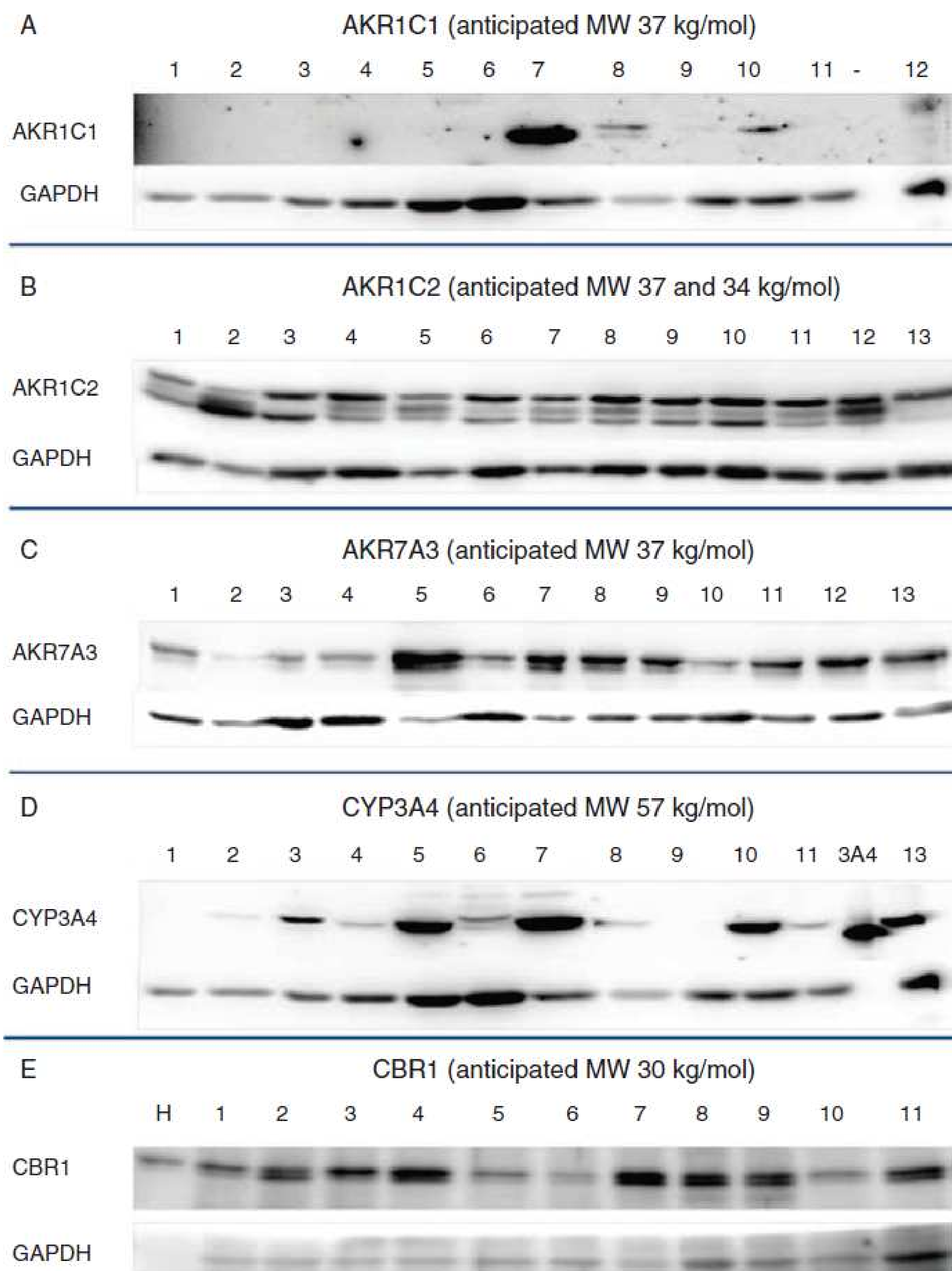

**C – ABCC8 (predicted MW 140 kDa)**




**D – ABCD2 (predicted MW 83 kDa)**

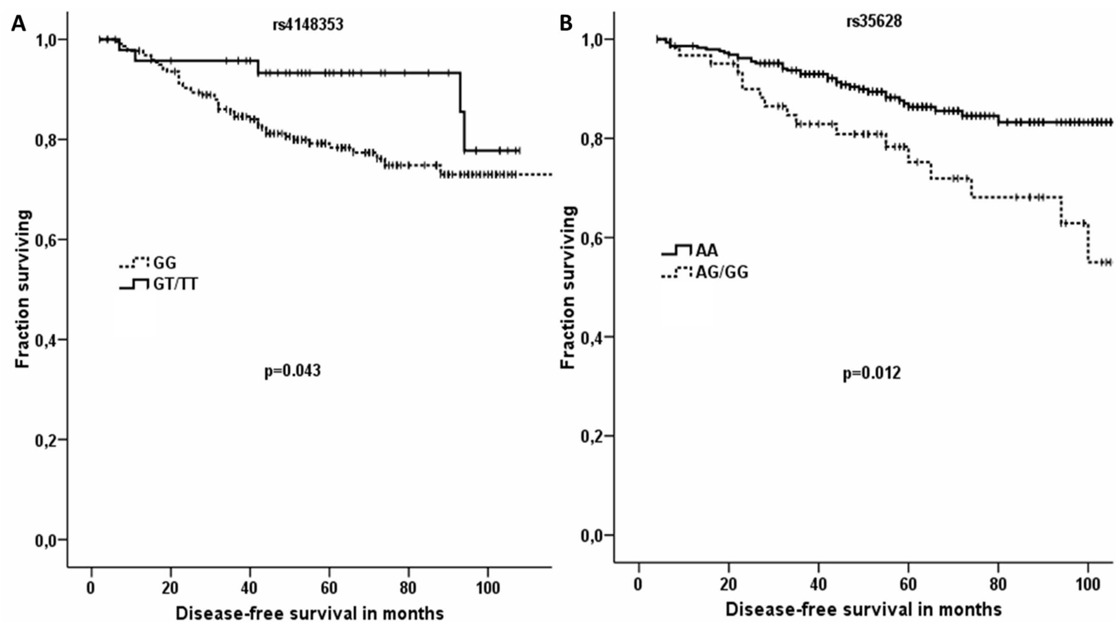



**Obrázek 7: Exprese proteinu ABC transportérů v nádorové tkáni.** Hladina proteinu byla detekována pomocí imunoblotingu. Kontrolní protein β-aktin byl použit jako kontrola nanášky a pro normalizaci hladin proteinů. Na obrázku je ukázán i odhad molekulové hmotnosti proteinu podle Human Protein Atlas (www.proteinatlas.org). Pozn.: MW = molekulová hmotnost, „molecular weight“



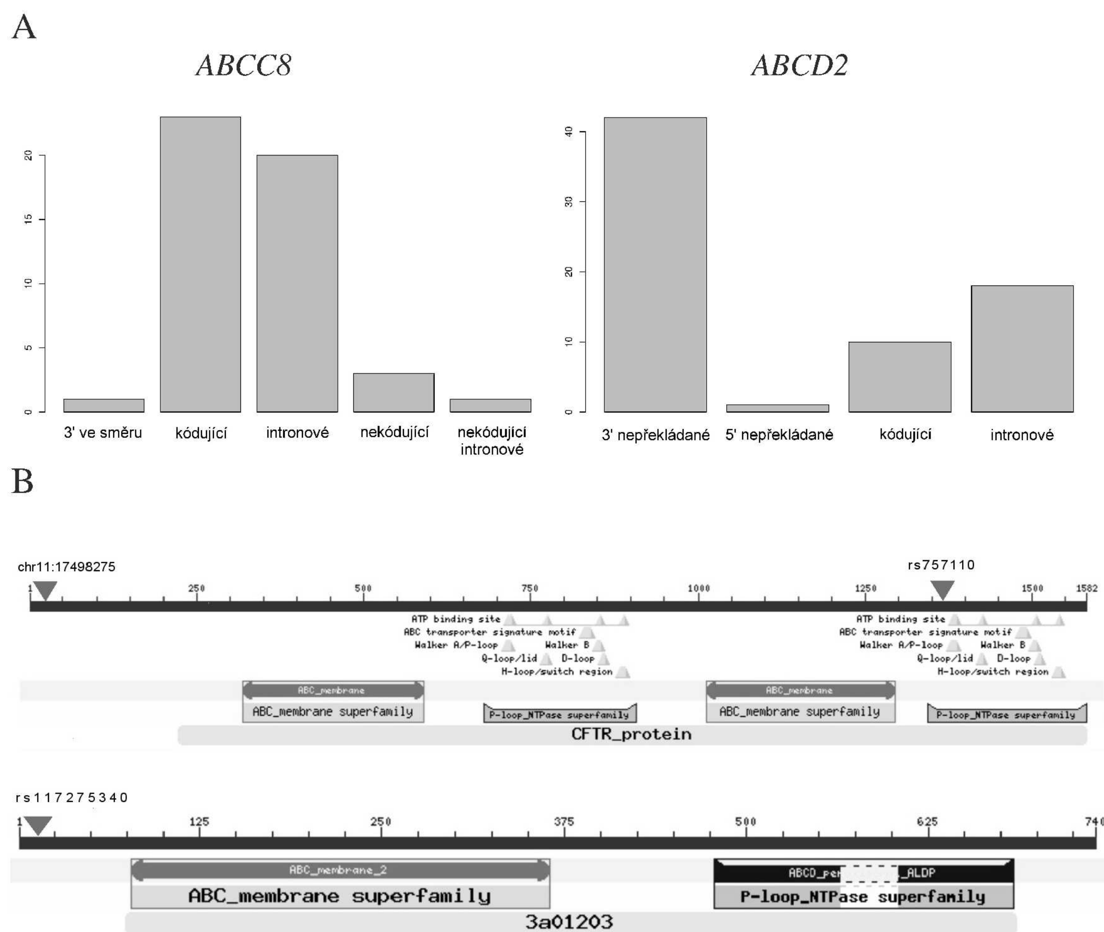

**Obrázek 8: Exprese vybraných kandidátních proteinů v nádorové tkáni pacientek.** Exprese vybraných proteinu byla hodnocena pomocí imunoblotingu. GAPDH byla použita jako kontrola nanášky a pro normalizaci hladin proteinů. Na obrázku je ukázán i odhad molekulové hmotnosti proteinu podle Human Protein Atlas (www.proteinatlas.org).

Pozn.: GAPDH = glyceraldehydfosfátdehydrogenáza; MW = molekulová hmotnost, „molecular weight“




**Obrázek 9: Významné vztahy mezi bezpříznakovým přežitím pacientek a SNP v genu *ABCC1*.** Kaplan-Meierovy křivky přežívání byly analyzovány pomocí Breslowova testu pro pacientky léčené pomocí chemoterapie (**A**) nebo hormonální terapie (**B**). **A)** Čárkovaná čára reprezentuje DFS pacientek s genotypem GG v polymorfismu rs4148353, plná čára značí pacientky s alelou T. **B)** Čárkovaná čára reprezentuje DFS pacientek s alelou G v polymorfismu rs35628 a plná čára znamená DFS pacientek s genotypem AA.

Pozn.: SNP = jednonukleotidový polymorfismus, „single nucleotide polymorphism“; DFS = přežití bez příznaků onemocnění, „disease-free survival“




**Obrázek 10: Rozložení alterací v genech *ABCC8* a *ABCD2*. A)** Četnost genetických alterací v genu *ABCC8* (vlevo) a v genu *ABCD2* (vpravo) analyzovaná podle serveru USCS (http://snp-nexus.org). Počty alterací jsou na ose y. **B)** Pozice kódujících jednonukleotidových polymorfismů (SNP) s predikovaným patogenním efektem v genu *ABCC8* (nahoře) a v genu *ABCD2* (dole) jsou označeny pomocí trojúhelníků. Schéma ABC domén je převzato z NCBI's Conserved Domain Database (Marchler-Bauer a kol. 2011).

# 7 Diskuse

Hlavním cílem personalizované terapie nádorových onemocnění je nalézt jednoduchý, cenově dostupný nástroj ke stanovení co nejúčinnější a zároveň nejbezpečnější individuální terapie pro pacienty. V této disertační práci jsem se zabýval hledáním kandidátních genů metabolismu a transportu cytostatik ve vztahu k léčbě a k prevenci resistence karcinomu prsu. Tato práce vychází z předpokladu, že je možné nalézt, validovat a určit mechanismus působení genů, které mohou ovlivňovat výsledek chemoterapie nebo prognózu pacientek, popř. spolupůsobit např. na expresi hormonálních receptorů. Cílem je, aby tyto kandidátní geny byly schopné spolehlivě predikovat výsledek terapie pacientek nebo aby alespoň byla prokazatelná souvislost mezi těmito geny a prognózou onemocnění těchto pacientek. Pokud bude mechanismus působení závislý na genetické výbavě, je možné prozkoumat funkční důsledky genetických variant a využít je pro predikci nejvhodnější léčby pacientek. Příkladem takového genu je KRAS a jeho využití pro predikci resistence kolorektálního karcinomu k protilátce cetuximab. Stanovení genetických polymorfismů je cenově a technicky dostupné a může být využito u velkého počtu pacientek. Na rozdíl např. od stanovení transkriptu metodami real-time PCR či mikročipů nebo stanovení proteinu imunochemicky, může být široce využito v panelech genů zařazených do běžného screeningu u karcinomu prsu (Lhota a kol. 2016).

Kandidátní geny (ABC transportéry, CYP a AKR) byly zkoumány pomocí kvantitativní real-time PCR (transkript), imunoblotingu (protein), přímého sekvenování a NGS (gen). Byl stanoven expresní profil genů v nádorové tkáni pacientek a porovnáván s jejich klinicko-patologickými daty. Potenciální biomarkery byly validovány pomocí imunoblotingu, ověřením na nezávislém souboru pacientek a stanovením mechanismu účinku, konkrétně hledáním genetických polymorfismů a vzácných variant v kandidátních genech pomocí sekvenování DNA z krve pacientek. Dalšími možnostmi zkoumání mechanismu účinku jsou např.: stanovení methylací genů, sledování mikroRNA, funkční stanovení aktivity enzymů v transportu a stanovení biotransformačního potenciálu metabolických genů v *in vitro* či *in vivo* modelech atd.

## *7.1 ABC transportéry*

Jedněmi z hlavních souvislostí odhalených v této práci jsou vztahy hladin transkriptů genů ABCA12, ABCA13 a ABCD2 k odpovědi na léčbu pacientek a dále vztahy genů ABCC1 a ABCC8 k expresi ER a PR a stupni vyzrávání (grade) nádoru.

Z hlediska statistické významnosti byla jedním z nejsilnějších nalezených vztahů asociace vyšších hladin transkriptu genu ABCC8 v nádorové tkáni u pacientek s pozitivní expresí ER a s nižším grade nádoru. Rovněž byl zaznamenán vztah hladin transkriptu s odpovědí na léčbu pacientek, ale tento vztah nebyl statisticky významný. Pacientky odpovídající na neoadjuvantní léčbu měly nižší hladiny ABCC8 než pacientky s progresivní nebo stabilní chorobou (hladina významnosti P = 0,096). Gen ABCC8 nekóduje efluxní pumpu, ale SUR1 – část $K^+$ kanálu regulovaného pomocí ATP. Pacienti s mutacemi v genu *ABCC8* trpí familiární hyperinsulinemickou hypoglykemií nebo naopak diabetes mellitus. Pacienti s diabetem jsou léčeni pomocí sulfonylmočoviny, která stimuluje funkci β-buněk pankreatu tím, že obchází metabolickou dráhu a uzavírá draslíkový kanál, čímž dojde následně k sekreci insulinu. V recentní literatuře byla popsána souvislost glukózového metabolismu s nádorovým onemocněním (Vander Heiden a kol. 2009). Vztah exprese genu ABCC8 s prognózou onemocnění, nalezený u našich dvou souborů pacientek před a po terapii, by tedy mohl být vysvětlen spíše fyziologickým efektem ABCC8 (např. souvislostí hladiny glukózy se vznikem a progresí nádorů) než působením produktu genu přímo na léčivo. Gen *ABCC8* jsme dále sledovali z hlediska komplexní genetické analýzy. Exony genu s přesahem 30 bází do přilehlých intronových oblastí byly prozkoumány pomocí cíleného obohacení DNA souborem prób Sequence Capture EZ Choice (Nimblegen, Roche) a sekvenovány pomocí NGS. Výsledek pilotní studie byl publikován (Souček a kol. 2015) a na jeho základě byl navržen panel kandidátních genů karcinomu prsu (n = 510 genů) s cílem provést analýzu u min 100 pacientek metodou sekvenování na přístroji MiSeq (Illumina Inc., San Diego, USA). Z výsledků publikované pilotní studie je nejzajímavější nález polymorfismu rs757110, 587C>A s predikovaným efektem na funkci proteinu i na vazbu transkripčních faktorů (hodnocení *in silico* v databázích PolyPhen-2

„Polymorphism phenotyping“ a RegulomeDB). Meta-analýzou bylo zjištěno, že alela C tohoto polymorfismu má vztah k náchylnosti k diabetu 2. typu (Qin a kol. 2013). Naším dalším cílem je ověřit její význam pro vznik a průběh karcinomu prsu na velkém historickém souboru pacientek (n > 800). Tato studie probíhá.

Dalším, námi nalezeným, kandidátním genem je ABCD2. Pacientky s částečnou nebo úplnou odpovědí na neoadjuvantní léčbu měly významně vyšší hladiny transkriptu ABCD2 než pacientky s nižšími hladinami a ve skupině adjuvantně léčených pacientek negativně korelovala hladina transkriptu s velikostí nádorů. Pacientky s hladinami ABCD2 vyššími než medián léčené neoadjuvantně měly také významně delší DFS (Hlaváč a Souček 2015; Obrázek 2). ABCD2 je adrenoleukodystrofický gen zodpovědný za transport lipidů do liposomů (Hlaváč a Souček 2015). V současné literatuře je popsán vztah mezi obezitou, zánětlivými procesy a nádorovým onemocněním (Liu a kol. 2012). V recentní práci se objevil zajímavý vztah diabetického léku metforminu a ABCD2. Metformin má indukční efekt na expresi ABCD2 u myší s X-vázanou adrenoleukodystrofíí, koriguje metabolickou funkci a snižuje zánětlivé procesy způsobené nedostatkem ABCD2. Mechanismem působení metforminu je aktivace proteinkinázy aktivované molekulou AMP (AMPK), přičemž AMPK pozitivně reguluje β-katenin (Singh a kol. 2016). Aktivace genu ABCD2 pomocí Wnt/β-kateninové dráhy byla již dříve popsána (Park a kol. 2013). Tato dráha souvisí se vznikem nádorů tlustého střeva a pankreatu (Bienz a Clevers 2000; Jones a kol. 2008). V této souvislosti je zajímavé, že metformin je stále častěji používán v adjuvantní léčbě nádorových onemocnění (Coyle a kol. 2016; Kim a kol. 2016; Rico a kol. 2016). Je také popsáno, že metformin snižuje hladiny estrogenů a androgenů u pacientek s karcinomem prsu (Campagnoli a kol. 2013). Efekt působení tohoto léku je u různých typů nádorů v současné době intenzivně zkoumán (Gadducci a kol. 2016), u karcinomu prsu je protinádorové působení zprostředkováno miR-26a (mikroRNA má za cíl geny PTEN a EHZ2; Cabello a kol. 2016). Transportér ABCD4 byl naší skupinou popsán jako kandidátní gen u kolorektálního karcinomu ve skupině pacientů léčených adjuvantně režimy obsahujícími 5-fluorouracil. Pacienti s nižšími hladinami ABCD4 v nádorech měly signifikantně kratší DFS než pacientky s vyššími hladinami (Hlavatá a kol. 2012). Tyto

poznatky naznačují možný význam ABC transportérů z rodiny D pro prognózu a pravděpodobně i predikci vzniku nádorového onemocnění obecně. Gen *ABCD2* byl rovněž sekvenován pomocí NGS v našem pilotním projektu. Byl nalezen polymorfismus rs117275340 u jedné pacientky, jedná se o nesynonymní záměnu aminokyselin A9G, která je predikována jako poškozující, tj. měnící funkci proteinu, podle *in silico* databází SIFT („Sorting intolerant from tolerant“) a PolyPhen-2). Tento polymorfismus byl ověřen pomocí přímého sekvenování DNA z krve pacientek s karcinomem prsu (nepublikované výsledky). Z tohoto pohledu se metoda NGS jeví jako slibná pro výzkum významu biomarkerů z rodiny ABC transportérů v prevenci nádorových onemocnění.

Dále byla, v naší studii, nalezena souvislost genové exprese ABCC1 s expresí hormonálních receptorů a se stupněm onemocnění pacientek. Pacientky s nádory bez exprese ER a s vyšším stupněm vyzrávání nádoru měly významně vyšší hladiny transkriptu ABCC1 v nádorech, než pacientky s nižším stupněm a s nádory exprimujícími ER. V naší studii zkoumající 12 polymorfismů v oblasti nukleotidové vazebné domény 1 genu *ABCC1* byl zaznamenán haplotyp rs35626-rs4148351, který souvisel se zvýšenou expresí genu v pořadí CT-GT>CC-GT>CC-GG a také vztah minoritní alely v polymorfismech rs35623 a rs35628 s vyšší expresí ABCC1. Dva polymorfismy rs4148353 a rs35628 signifikantně ovlivňovaly DFS. Nosičky minoritní alely T v polymorfismu rs4148353 měly signifikantně delší přežívání než pacientky genotypu AA. Pacientky s minoritní alelou G polymorfismu rs35628 měly signifikantně kratší přežívání než pacientky genotypu AA. *ABCC1*/MRP1 je velmi často studovaným membránovým transportérem (Kunická a Souček 2014). Jeho význam pro mnohočetnou lékovou resistenci byl již prokázán u různých nádorů *in vitro* i *in vivo*. Nyní je aktuální výzvou nalezení genetických polymorfismů, epigenetických faktorů a mechanismů účinku, které by mohly mít vliv na chemoresistenci, a jinými slovy mohly být využity k ovlivnění sensitivity různých nádorů k léčivům (Spitzweiser a kol. 2016; Lu a kol. 2015).

## *7.2 Aldoketoreduktázy a karbonylreduktáza*

V rodině AKR byly, v naší studii, identifikovány tři potenciální markery. Pacientky s expresí hormonálních receptorů v nádorech měly významně vyšší hladinu genu AKR7A3 než pacientky bez exprese ER a PR. Adjuvantně léčené pacientky s vyšší hladinou AKR7A3 než medián, měly signifikantně delší DFS oproti pacientkám s nižšími hladinami. O vztahu k nádorovým onemocněním je v literatuře velmi málo informací. Rezaul a kol. (2010) pozoroval vyšší expresi proteinu AKR7A3 ve vzorcích od pacientek s ER pozitivním karcinomem prsu. U hepatocelulárního karcinomu byla vyšší exprese AKR7A3 spojena s delším OS, v podrobné studii bylo prokázáno, že buňky hepatocelulárního karcinomu HCC exprimující AKR7A3 mají oproti buňkám s umlčeným genem AKR7A3 sníženou tumorogenitu a chemoresistenci, mechanismem je oslabení drah ERK, c-Jun a NF-κB (Chow a kol. 2016). Albrethsen a kol. (2011) naopak pozoroval zvýšenou expresi proteinu AKR7A3 v hepatomech oproti kontrolám na modelu potkanů s resistentními hepatocelulárními nádory jater.

Enzymy AKR1C1 a AKR1C2 hrají důležitou úlohu v metabolismu steroidních hormonů, což vytváří předpoklad, že by mohly být účastny v proliferaci nádorů (Zhao a kol. 2015). V naší studii byla nalezena negativní korelace exprese AKR1C1 s velikostí nádoru, vztah mezi vyššími hladinami AKR1C2 a delším DFS neoadjuvantně léčených pacientek a vztah vyšších hladin AKR1C1 a AKR1C2 v nádorech s dobrou odpovědí na neoadjuvantní léčbu. Tento výsledek je ve shodě se studií, ve které byla nalezena korelace vyšší imunohistochemické hladiny proteinů AKR1C1 a AKR1C2 s delším DFS a OS pacientek s karcinomem prsu, menší velikostí nádoru a s výskytem lymfatických uzlin bez zasažení nádorovým procesem (Wenners a kol. 2016). Odpověď na léčbu však v této práci sledována nebyla. Je známo, že AKR1C1 metabolizuje progesteron na 20α-hydroxyprogesteron a AKR1C2 na 3α-hydroxyprogesteron. Tento efekt, který vede k inaktivaci progesteronu a k potlačení proliferace nádoru, je pravděpodobně zodpovědný za prodlouženou dobu přežití a lepší prognózu pacientek (Wenners a kol. 2016), avšak v jiné práci je popsáno, že zánětlivý interleukin-1β zvyšuje hladinu AKR1C1 u buněčných linií karcinomu močového

měchýře, přičemž umlčení genu AKR1C1 snižuje invasivitu a chemoresistenci těchto linií (Matsumoto a kol. 2016).

Pacientky s nejvíce agresivními nádory (grade 3) měly, v naší studii, významně vyšší hladiny transkriptu CBR1 v nádorech než pacientky s grade 1 nebo 2. V podskupině pacientek léčených hormonálně měly pacientky s vyššími hladinami CBR1 významně kratší DFS než pacientky s nižšími hladinami. Naopak v podskupině pacientek léčených pouze chemoterapií měly pacientky s vyššími hladinami CBR1 delší DFS než pacientky s nižšími hladinami. Tento ambivalentní vztah je pravděpodobně zapříčiněn malým vzorkem pacientek ve skupinách rozdělených podle terapie (n = 25 ve skupině s chemoterapií, respektive n = 23 ve skupině hormonálně léčených pacientek). Řada jiných studií podporuje výsledek nepříznivé prognózy vyšší hladiny CBR1. Polymorfismy CBR1 a CBR3 u pacientek s karcinomem prsu ovlivňují plasmovou clearance adriamycinu a tím i expozici cytostatiku (Lal a kol. 2008). Inhibice CBR1 zlepšuje účinnost chemoterapie založenou na doxorubicinu na zvířecím modelu (Jo a kol. 2017). Ve studii provedené na buněčné linii MCF-7 byla hladina CBR1 indukována hypoxií s následnou resistencí na doxorubicin. Resistence může být zvrácena umlčením genu CBR1 nebo faktoru indukovatelného hypoxií (HIF) -1α. CBR1 i HIF-1α mohou být inhibovány také resveratrolem, známým inhibitorem *ABCC1*/MRP1 (Mitani a kol. 2014). Na druhou stranu je popsán i inhibiční efekt CBR1 na růst nádoru a proapoptotické působení CBR1 u karcinomu ovaria (Miura a kol. 2015). Navíc byl pozorován prognostický vztah snížené exprese proteinu CBR1 ke kratšímu DFS a OS u karcinomu endometria (Murakami a kol. 2012). CBR1 také působí protektivně jako antioxidant a je indukována transkripčním faktorem Nrf2 (Miura a kol 2013). Dle současné literatury CBR1 vyvolává apoptózu vazbou na receptor tumor nekrotizujícího faktoru (TNF); zvýšená exprese CBR1 snižuje hladiny NF-κB a protoonkogenu c-Jun (Miura a kol. 2015) a může tak posilovat účinky podané chemoterapie. V každém případě jak AKR, tak CBR patří k poměrně vzácně studovaným enzymům v karcinogenezi, avšak naše studie jednoznačně ukazuje, že je třeba jejich úlohu v rozvoji a progresi nádorových onemocnění dále sledovat.

## *7.3 Cytochromy P450*

CYP patří mezi nejčastěji studované enzymy metabolismu léčiv v preventivní medicíně a zejména v experimentální farmakologii a onkologii. V naší studii měly pacientky s hladinami CYP3A4 vyššími než medián signifikantně delší DFS než pacientky s nižšími hladinami. Souvislost CYP3A4 a některých dalších CYP s rizikem vzniku nádorů nebo s nepříznivým výsledkem léčby karcinomu prsu je v literatuře poměrně dobře popsána (Miyoshi a kol. 2009; Miyoshi a kol. 2005), stejně jako souvislost s metabolismem steroidních hormonů – CYP3A4 se v játrech podílí na aktivaci estronu, zvláště potentního estrogenu, na 2-hydroxyestradiol s karcinogenními účinky (Shou a kol. 1997). Bylo zjištěno, že genetické varianty CYP3A, např. SNP rs10235235, mají vztah k metabolismu estrogenu i k věku při menarche a také k riziku vzniku karcinomu prsu (Johnson a kol. 2014). Některé CYP včetně CYP3A4 jsou spojené s horším OS pacientek s karcinomem prsu (Murray a kol. 2010). Na druhou stranu fakt, že CYP3A4 aktivuje cyklofosfamid (Roy a kol. 1999; Chang a kol. 1993) by mohl vysvětlovat námi nalezený vztah vyšších hladin CYP3A4 s delším DFS pacientek neoadjuvantně léčených cyklofosfamidem. Z těchto výsledků vyplývá, že působení CYP3A4 na prognózu je spíše nejasné, ale v literatuře je dostatečně popsána souvislost s prognózou i s predikcí vzniku karcinomu prsu.

Dalším, námi odhaleným, kandidátním genem je CYP2B6. Pacientky s expresí ER a PR v nádorech měly významně vyšší hladiny CYP2B6 v nádorové tkáni než pacienty bez exprese. Pacientky s vyššími hladinami transkriptu CYP2B6 než medián měly navíc významně delší DFS než pacientky s nižšími hladinami. V předchozích studiích byla pozorována nadměrná exprese genu CYP2B6 na úrovni mRNA v nádorech prsu pacientek s expresí ER v porovnání s nádory bez exprese ER a také v porovnání s normální prsní tkání (Tozlu a kol. 2006; Bieche a kol. 2004). Naše studie tedy tento výsledek validovala na nezávislém souboru pacientek. Tento efekt je také potvrzen *in vitro* ve studii provedené na lidské buněčné linii karcinomu prsu T-47D. ER zvyšuje expresi CYP2B6 regulací ERE v promotorové oblasti genu *CYP2B6* (Lo a kol. 2010). Exprese CYP2B6 je spolu s CYP3A4 regulována Wnt/β-kateninovou drahou (Thomas a kol. 2015), která souvisí se vznikem nádorů (Bienz a Clevers 2000;

Jones a kol. 2008), a tak by mohla vysvětlovat úlohu těchto genů v karcinogenezi. CYP2B6 spolu s CYP2D6 hrají spíše vedlejší úlohu v aktivaci prokarcinogenů, a i když je vliv polymorfismů *CYP2B6* na metabolismus cyklofosfamidu u pacientek s karcinomem prsu spíše minoritní (Jamieson a kol. 2014), v několika současných studiích byl popsán vliv SNP na délku přežívání pacientek. U pacientek s karcinomem prsu léčených cyklofosfamidem byly zkoumány nesynonymní SNP 785A>G (rs2279343, *4), 1459C>T (rs3211371, *5) a 516G>T (rs3745274, *9), které mění aminokyselinové pořadí v proteinu a snižují funkci enzymu. Haplotypem CYP2B6*6 jsou označovány pacientky, jež nesou současně variantní alely *CYP2B6* 785A>G (rs2279343, *4) a 516G>T (rs3745274, *9). Haplotypy *5/*6, *6/*9 nebo *6/*6 signifikantně asociovaly s kratším DFS pacientek (Haroun a kol. 2015). CYP2B6*5 je prediktorem špatné odpovědi pacientek s karcinomem prsu na neoadjuvantní léčbu cyklofosfamidem (Tulsyan a kol. 2014). V případě studií na pacientkách léčených jinou terapií než režimy založenými na cyklofosfamidu jsou výsledky odlišné. CYP2B6*6 nemá vliv na DFS pacientek s karcinomem prsu léčených tamoxifenem (Mwinyi a kol. 2014). V jiné studii provedené na pacientkách s metastazujícím karcinomem prsu, léčených docetaxelem a thiotepou, měly pacientky s genotypem AG v polymorfismu rs2279343 delší OS (Song a kol. 2015). Byl popsán i vztah polymorfismů v genu *CYP2B6* s rizikem vzniku karcinomu prsu. Genotypy GG a TT polymorfismů 785A>G (rs2279343), respektive 516G>T (rs3745274) zvyšují riziko vzniku karcinomu prsu, pravděpodobně zvýšením hladin endogenních estrogenů vlivem snížené aktivity enzymu CYP2B6 (Justenhoven a kol. 2014). Z uvedeného vyplývá, že CYP2B6 může hrát důležitou úlohu v léčbě karcinomu prsu cestou aktivace cyklofosfamidu a je proto vhodné dále prozkoumat úlohu tohoto genu v predikci léčby karcinomu prsu.

Díky efektivní aktivaci proléčiv je, zejména u karcinomu kolorekta, CYP2W1 slibným cílem vývoje nových terapeutik (Guo a kol. 2016). Ve shodě s touto hypotézou měly pacientky s karcinomem prsu, dobře odpovídající na léčbu, v naší studii významně vyšší hladiny transkriptu CYP2W1 než pacientky se stabilním nebo progresivním onemocněním. Naopak exprese proteinu CYP2W1 detekovaná imunohisto-chemicky je u kolorektálního karcinomu negativním

prognostickým markerem OS pacientů (Stenstedt a kol. 2012; Edler a kol. 2009). Jeho exprese je specifická pro daný typ nádoru. Na úrovni mRNA byla nalezena exprese především v nádorech nadledvin, tlustého střeva a v buněčné linii lidského hepatomu HepG2; naopak ve tkáních mozku, srdce, žaludku, střeva, ledvin, tlustého střeva, svalu, krve, placenty, močového měchýře, dělohy, nadledvin a štítné žlázy žádná exprese nalezena nebyla (Karlgren a kol. 2006). Se vznikem nádorového onemocnění může souviset také účast CYP2W1 v metabolismu mastných kyselin a lysofosfolipidů (Xiao a Guengerich 2012), neboť metabolismus lipidů se na vzniku nádorových onemocnění obecně podílí (Liu a kol. 2012). Přesto, že tento gen není u nádorových onemocnění zatím příliš prostudován a jeho role se zmiňuje spíše v souvislosti s kolorektálním karcinomem, naše studie ukázala na jeho možný potenciál jako biomarkeru odpovědi na chemoterapii u karcinomu prsu.

# 8 Závěr

V této práci byla objevena řada vztahů mezi hladinami transkriptů genů metabolismu a transportu léčiv a významnými klinickými prognostickými nebo prediktivními faktory karcinomu prsu. Také byly objeveny genetické varianty ve vybraných potenciálních biomarkerech prognózy a odpovědi na léčbu pacientek s karcinomem prsu. Výsledky naznačují, že uvedené kandidátní geny se mohou stát využitelnými biomarkery v prevenci progrese a selhání léčby nádorových onemocnění. Tyto výsledky je však ještě třeba ověřit, nejlépe validovat v nezávislých studiích. V České Republice byl již vytvořen panel genů významných pro riziko vzniku karcinomu prsu pacientek bez známých mutací v genech BRCA založený na cíleném NGS (CZECANCA: Czech cancer panel for clinical application), který cílí na 219 genů spojovaných s dědičnými nádorovými onemocněními (Soukupová a kol. 2016). Tento panel je zamýšlen pro přímé využití v aplikované vědě. Cílem je: „…zlepšení klinické diagnostiky dědičných nádorových onemocnění, které by mělo přinést zlepšení péče o nosiče mutací v nádorových predispozičních genech.“ V mé disertační práci byly objeveny některé geny z oblasti biologie mastných kyselin a sacharidů, které by mohly ovlivňovat prognózu nebo odpověď na léčbu pacientek s karcinomem prsu, a geny, které souvisejí s metabolismem či signálními drahami steroidních hormonů a mají přímou souvislost s přežíváním pacientek. U dalších genů je fyziologický efekt působení na prognózu či výsledek terapie spíše nejasný. Objevili jsme také množství genetických polymorfismů, které by mohly přispět k objasnění mechanismu účinku těchto kandidátních markerů. Jedním ze závěrů této práce, který lze ihned použít k dalšímu výzkumu v oblasti preventivní medicíny, je potřeba zařazení významných genů do panelu využitelného pro odhad prognózy a léčebné odpovědi u pacientek s karcinomem prsu s pomocí stejné metody jako je tomu u výše jmenovaného panelu pro screening genetické predispozice nádorům obecně.

V naší laboratoři v současné době probíhá výzkum epigenetických faktorů (methylace promotorových oblastí a stanovení kandidátních mikroRNA) vybraných genů v návaznosti na výsledky této disertační práce. Podle mého

názoru, v případě ověření klinického významu i funkčních aspektů některých zde popsaných markerů, bude možné tyto poznatky využít nejen k prevenci progrese a selhání léčby, ale rovněž jako cílů pro vývoj nové léčby.

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# 10 Seznam obrázků

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2. Vztah genové exprese ABCD2 k bezpříznakovému přežití neoadjuvantně léčených pacientek
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## *Komentované publikované práce*

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# Příloha č. 1:

Soucek P, **Hlavac V**, Elsnerova K, Vaclavikova R, Kozevnikovova R, Raus K.

**Whole exome sequencing analysis of ABCC8 and ABCD2 genes associating with clinical course of breast carcinoma**

Physiol Res. 2015;64 Suppl 4:S549-557 [IF: 1,487] 50 %*)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

*Physiol. Res. 64 (Suppl. 4): S549-S557, 2015*

# Whole Exome Sequencing Analysis of *ABCC8* and *ABCD2* Genes Associating With Clinical Course of Breast Carcinoma

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Received September 17, 2015
Accepted October 2, 2015

**Summary**

The aim of the present study was to introduce methods for exome sequencing of two ATP-binding cassette (ABC) transporters ABCC8 and ABCD2 recently suggested to play a putative role in breast cancer progression and prognosis of patients. We performed next generation sequencing targeted at analysis of all exons in *ABCC8* and *ABCD2* genes and surrounding noncoding sequences in blood DNA samples from 24 patients with breast cancer. The revealed alterations were characterized by *in silico* tools. We then compared the most frequent functionally relevant polymorphism rs757110 in *ABCC8* with clinical data of patients. In total, the study identified 113 genetic alterations (>70 % novel ones) in both genes. Of these alterations, 83 were noncoding, 13 synonymous, 10 frameshifts and 7 were missense alterations. Four *in silico* programs predicted pathogenicity of two polymorphisms and four newly identified alterations. Rs757110 polymorphism in *ABCC8* did not significantly associate with clinical data of the patients. In conclusion, exome sequencing identified several functionally relevant alterations in *ABCC8* and *ABCD2* genes that may further be used for a larger follow-up study aiming to assess their clinical significance.

**Key words**

ABC transporter • Breast • Cancer • Therapy • Next generation sequencing

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## Introduction

Breast cancer is the most common cancer in women and caused 471,000 deaths worldwide in 2013 (Global Burden of Disease Cancer Collaboration 2015). A number of cellular processes that in some cases lead to the tumor resistance limits efficacy of breast cancer therapy. Multidrug resistance (MDR) to a variety of chemotherapy drugs presents one of these processes (Baguley 2010).

MDR is often caused by the decreased cellular uptake or increased efflux of drugs and by alterations in DNA repair and apoptotic pathways. Drug efflux is mediated by membrane-bound ATP-binding cassette (ABC) transporters (Sakacs *et al.* 2006). ABC transporter family in humans consists of 48 genes and one pseudogene (Dean *et al.* 2001). ABCs translocate a wide variety of substrates, including lipids, sterols and drugs across extra- and intracellular membranes (Klaassen and Aleksunes 2010). Therefore, the most prominent ABCs as ABCB1/P-glycoprotein (Wolf *et al.* 2011), ABCC1/MRP1 (Kunicka and Soucek 2014), and ABCG2/BCRP (Natarajan *et al.* 2012), known to transport plethora of anticancer drugs belong to the most studied in cancer pathophysiology.

Our recent studies have shown that gene expression levels of a number of ABCs significantly differ

PHYSIOLOGICAL RESEARCH • ISSN 0862-8408 (print) • ISSN 1802-9973 (online)
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Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

between the tumor and paired non-malignant tissues from patients with solid tumors (colorectal – Hlavata *et al.* 2012, breast – Hlavac *et al.* 2013, pancreas – Mohelnikova-Duchonova *et al.* 2013) suggesting their potential role in cancer progression. Moreover, tumor levels of some ABCs were associated with clinical characteristics of the patients including prognostic factors (e.g. the expression of estrogen receptor with ABCC1 and ABCC8 in breast cancer or grade with ABCC10 in colorectal cancer). Most interestingly, intratumoral ABCs levels were associated with the response to neoadjuvant chemotherapy in breast cancer (Hlavac *et al.* 2013) and survival of the patients in colorectal cancer (Hlavata *et al.* 2012). Particularly, observed significant overexpression of ABCD2 in tumor tissues of breast cancer patients with partial or complete response (responders) after the neoadjuvant chemotherapy in comparison to patients with stable or progressive disease (non-responders) attracts our attention (Hlavac *et al.* 2013). The revealed existence of a broad variability in protein expression of ABCC8 (OMIM: 600509) and ABCD2 (OMIM: 601081) between tumor samples (Hlavac *et al.* 2013) further suggests that expression changes of ABCs levels could be biologically relevant for breast cancer.

Additionally, our most recent data showed that genetic variability in candidate marker *ABCC1* associates with its gene expression in tumor tissue and with survival of breast cancer patients (Kunicka *et al.* 2014) providing a proof-of-principle for further explorations.

Thus, recent studies demonstrate that phenotype and genotype of genes associated with MDR, namely ABCs, could be useful for individualization of cancer therapy. The aim of our present study is to expand the present knowledge about genotype of the most interesting ABCs for breast cancer prognosis and therapy outcome. Thus, here we explored genetic variability of candidate ABC transporters (namely *ABCC8* and *ABCD2*) in peripheral blood DNA samples from breast cancer patients by exome sequencing and predicted functional consequences of the identified alterations on their phenotypes. The most interesting alterations may now be used for a large scale follow-up study targeted at evaluation of their functional, prognostic, and predictive potential.

## Patients and Methods

*Patients*

The study included a total of 24 breast cancer patients (C50 according to the International Classification of Diseases, the 10th revision) of Caucasian origin diagnosed in Institute for the Care for Mother and Child and the Department of Oncosurgery, Medicon, in Prague during 2006-2012. Patients underwent preoperative neoadjuvant chemotherapy regimens based on 5-fluoro-uracil/anthracyclines/cyclophosphamide (FAC or FEC) and/or taxanes. The collection of blood samples, and the retrieval of clinical data were performed as described before (Vaclavikova *et al.* 2012). Following data on patients were retrieved from medical records: age at diagnosis, menopausal status, personal medical history, family history (number of relatives affected by breast/ovarian carcinoma or other malignant diseases), stage, tumor size, presence of lymph node metastasis, histological type and grade of the tumor, expression of estrogen, progesterone and ERBB2 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2) receptors, expression of Ki67, response to the therapy and progression-free survival. All patients after primary chemotherapy and surgery were followed for local or distant relapse or in the case of palliative setting for disease progression by regular medical visits every 3 months during the first 3 years, twice a year during the next 2 years and yearly then after. During the visits, mammography, chest X ray, skeletal survey, and abdominal ultrasound were performed yearly. Clinical examination together with tumor markers (CEA and CA 15-3) was performed during every visit. In the case of clinical uncertainty, additional tests and examinations were performed to rule out possible disease relapse or progression. Clinical characteristics of patients are presented in Table 1.

All subjects were informed and gave their written consent to participate in the study. The design of the study was approved by the Ethical Committee of the National Institute of Public Health in Prague.

*DNA extraction*

Blood samples were collected during the diagnostic procedures using tubes with $K_3$EDTA anticoagulant. Peripheral blood lymphocytes were prepared by using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) from fresh blood samples of patients. Genomic DNA was isolated from human peripheral blood lymphocytes by the standard phenol/chloroform extraction and ethanol precipitation method (Topic and Gluhak 1991). DNA samples were stored in aliquots at –20 °C prior to analysis.

**Table 1.** Clinical characteristics of patients.

| Clinical characteristics | | N | % |
|---|---|---|---|
| *Age at diagnosis (years)* | | 48.9 ± 10.8 | |
| *Menopausal status* | *Premenopausal* | 13 | 54 |
| | *Postmenopausal* | 11 | 46 |
| *Tumor size (mm)* | | 22.8 ± 13.4 | |
| *Lymph node metastasis* | *Absent (pN0)* | 16 | 67 |
| | *Present (pN1-3)* | 8 | 33 |
| *Pathological stage* | *I* | 9 | 41 |
| | *II* | 11 | 50 |
| | *III* | 2 | 9 |
| | *IV* | 0 | - |
| | *Not determined* | 2 | - |
| *Histological type* | *Invasive ductal carcinoma* | 19 | 79 |
| | *Other type* | 5 | 21 |
| *Pathological grade* | *1* | 3 | 13 |
| | *2* | 9 | 39 |
| | *3* | 11 | 48 |
| | *Gx* | 1 | - |
| *Estrogen receptor status* | *Positive* | 18 | 75 |
| | *Negative* | 6 | 25 |
| *Progesterone receptor status* | *Positive* | 18 | 75 |
| | *Negative* | 6 | 25 |
| *Expression of HER2* | *Positive* | 8 | 65 |
| | *Negative* | 15 | 35 |
| | *Not determined* | 1 | - |
| *Expression of Ki-67 (%)* | | 37.2 ± 24.5 | |
| *Response to neoadjuvant chemotherapy* | *Complete or partial response* | 11 | 48 |
| | *Stable disease or progression* | 12 | 52 |
| | *Not determined* | 1 | - |
| *Distribution of ABCC8 rs757110 allele frequencies* | CC | 4 | 17 |
| | CA | 9 | 38 |
| | AA | 11 | 46 |

Data are mean ± SD.

*Exome sequencing*

Libraries encompassing all exons of *ABCC8* (39 exons) and *ABCD2* (10 exons) genes were prepared according to the manufacturer (Roche, Prague, Czech Republic). Based on the character of probe design, i.e. tiling; the exons were surrounded by approximately 30 bp regions of intronic sequences which were also sequenced in both directions. Target enrichment was performed using SeqCap EZ Choice by Nimblegen. Libraries were prepared using Rapid Library Preparation Kit (Roche). Samples were sequenced on 454 GS Junior system (Roche).

*Data analysis*

Raw data were processed by pipeline software Sequence Pilot (JSI Medical Systems, Ettenheim,

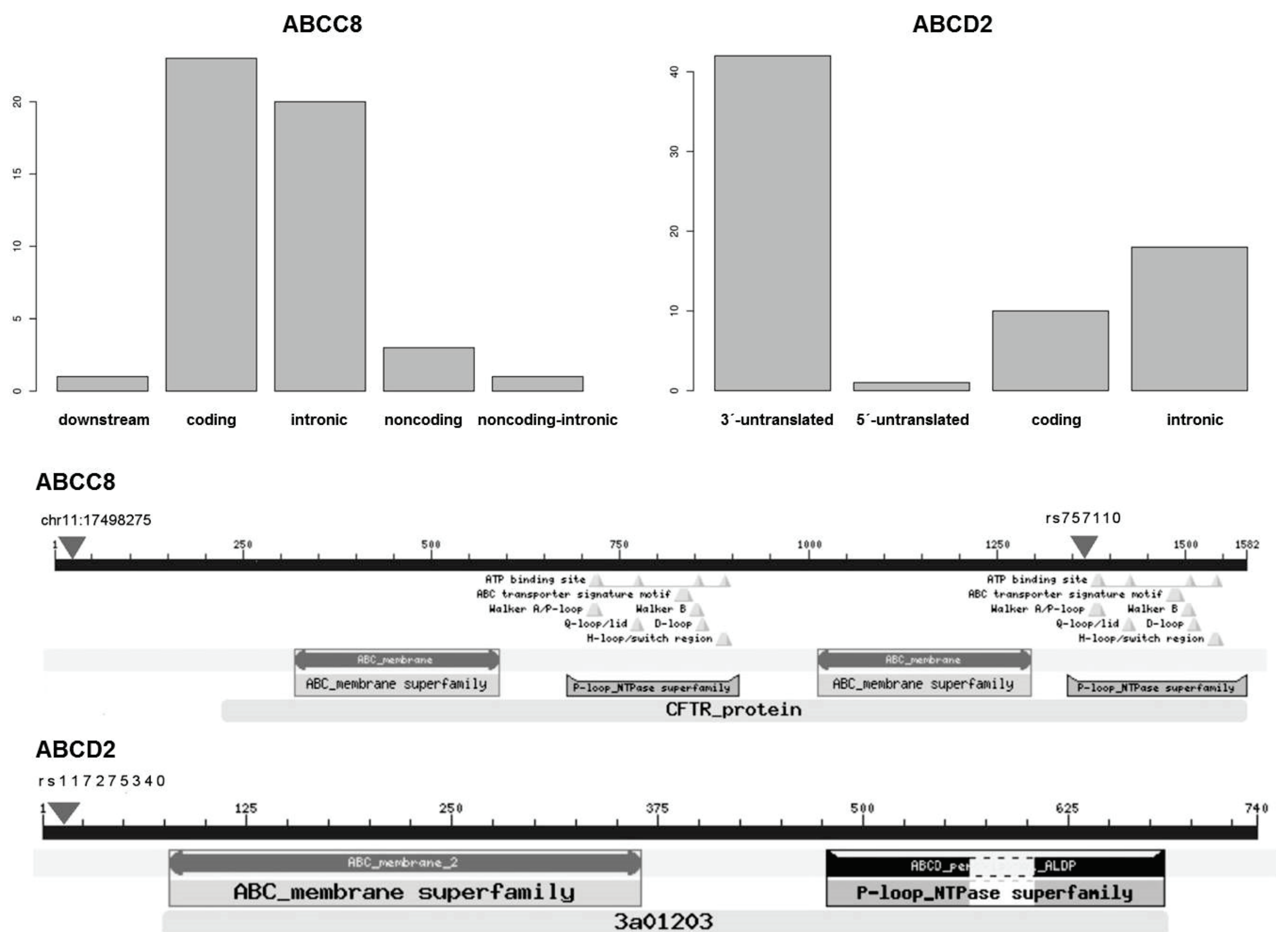


**Fig. 1.** Distribution of alterations in *ABCC8* and *ABCD2* genes. **Top**: The frequency of genetic alterations in ABCC8 (left) and ABCD2 (right) analyzed by the USCS server (http://snp-nexus.org). Numbers of alterations are on y-axis. **Bottom**: The positions of coding SNPs with the predicted pathogenic effects in *ABCC8* (upper image) and *ABCD2* (lower image) genes are depicted by triangles. The schematic presentation of ABC domains is adopted from NCBI's Conserved Domain Database (Marchler-Bauer *et al.* 2011).

**Table 2.** Overview of identified alterations in *ABCC8* and *ABCD2* genes in breast cancer patients.

| Type | *ABCC8*[a] | *ABCD2*[a] | Total[a] |
|---|---|---|---|
| *Noncoding* | 21 (1) | 62 (2) | 83 (3) |
| *Frameshift* | 6 | 4 | 10 |
| *Missense* | 5 (2) | 2 (1) | 7 (3) |
| *Synonymous* | 10 | 3 | 13 |
| *All* | 41 (3) | 72 (3) | 113 (6) |

Numbers of alterations with numbers of pathogenic ones in parentheses, [a] Pathogenic by Regulome DB, SIFT, PolyPhene or HaploReg (see Table 3).

Germany) and variant calling was performed with the following settings: minimal absolute coverage, 15-combined; both directions minimal absolute coverage, off; minimal % coverage, 10 % per direction.

Associations of single nucleotide polymorphisms (SNPs) and novel pathogenic alterations with prognostic clinical data (tumor size and grade, presence of lymph node metastasis, expression of hormonal receptors, ERBB2 and Ki67, progression-free survival, and response to the therapy) were evaluated by the two-sided Pearson chi square and the Spearman tests. A p-value of less than 0.05 was considered statistically significant. Analyses were conducted by the statistical program SPSS v15.0 (SPSS, Chicago, IL).

The functional relevance of the examined SNPs was analyzed *in silico* by Regulome DB (http://regulome.stanford.edu) (Boyle *et al.* 2012), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.jcvi.org) and HaploReg v2 and v3 (Ward and Kellis 2012) programs.

## Results

*Exome sequencing of ABCC8 and ABCD2 in breast cancer patients*

The 24 samples were sequenced with a mean coverage 87. Of the total number 49 regions, 100 % base

pairs were called in 42 regions, three regions were covered by >99 %, four by <80 % and one region (exon 1 in *ABCC8*) was covered by less than 50 %. In the two genes (*ABCC8* and *ABCD2*), 113 genetic alterations were identified, of which 83 alterations were in noncoding regions and the rest in the coding regions. Thirteen coding alterations were synonymous, seven missense amino acid changes and the rest of the identified alterations (n=10) were frameshift mutations present in three patients of the 24. Together, 81 (72 %) novel alterations (20 of 41, i.e. 50 % in *ABCC8* and 61 of 72, i.e. 85 % in *ABCD2*) were discovered. The observed genetic alterations are summarized in Table 2. Figure 1 (top part) shows the distribution of genetic alterations by type.

*Functional aspects*

*In silico* analysis by four different programs was used to predict functional relevance of all identified alterations. SIFT and/or PolyPhen and Regulome DB predicted probably deleterious effect of polymorphisms rs757110 in *ABCC8* and rs117275340 in *ABCD2*. The analysis by HaploReg suggested that these SNPs change binding sites for a number of transcription factors. One novel SNP (V17M) and one insertion in *ABCC8* and two insertion/deletions in *ABCD2* were predicted by the Regulome DB, SIFT and PolyPhen as deleterious (Table 3). Figure 1 (bottom part) depicts positions of coding alterations with the predicted pathogenic effects.

**Table 3.** *In silico* functional analysis of alterations in *ABCC8* and *ABCD2* genes revealed by the study.

| Coordinate | SNP ID | Amino acid change | Regulome DB | SIFT[a] | PolyPhen[b] | HaploReg[c] |
|---|---|---|---|---|---|---|
| ***ABCC8*** | | | | | | |
| chr11:17498225InsC | novel | none | Likely to affect binding | N/A | N/A | N/A |
| chr11:1749826C>T | novel | V17M | N/A | 0.02 | 0.984 | N/A |
| chr11:17418476C>A | rs757110 | A197S | Likely to affect binding and linked to expression of a gene target | N/A | 0.681 | CCNT2 SZF1-1 |
| ***ABCD2*** | | | | | | |
| chr12:39947545Delins3 | novel | none | Likely to affect binding | N/A | N/A | N/A |
| chr12:39947547DelA | novel | none | Likely to affect binding | N/A | N/A | N/A |
| chr12:40013392G>C | rs117275340 | A9G | N/A | 0.023 | 0.84 | AP-4_1 AP-4_3 Ascl2 CTCF_disc10 E2A_3 GATA_disc4 AP-4_3 Ascl2 CTCF_disc10 E2A_3 GATA_disc4 HEN1_1 HEN1_2 LBP-1_2 Myf_2 Myf_3 SREBP_known4 TCF12_known1 |

[a] score cutoff <0.05; [b] score cutoff >0.2; [c] motif changed according to HaploReg v3; *in silico* predictions for novel variants are not available (N/A). Missense alterations in grey.

**Table 4.** Distributions of pathogenic alterations in *ABCC8* and *ABCD2* genes in breast cancer patients.

| Patient no. | *ABCC8* | | | *ABCD2* | | |
|---|---|---|---|---|---|---|
| | **chr11:17418477C>A** | **chr11:17498275C>T** | **chr11:17498225InsC** | **chr12:39947545Delins3** | **chr12:39947547DelA** | **chr12:40013392G>C** |
| *P3* | CA | CC | -/- | -/- | -/- | GG |
| *P8* | AA | CC | -/- | -/- | -/- | GG |
| *P9* | AA | CC | -/- | -/- | -/- | GG |
| *P13* | AA | CC | -/- | -/- | -/- | GG |
| *P31* | AA | CC | -/- | -/- | -/- | GG |
| *P32* | AA | CC | -/- | -/- | -/- | GG |
| *P40* | CA | CC | -/- | -/- | -/- | GG |
| *P45* | CA | CC | -/- | -/- | -/- | GG |
| *P52* | CA | CC | -/- | -/- | -/- | GG |
| *P55* | AA | CC | -/- | -/- | -/- | GG |
| *P67* | AA | **CT** | **insC/-** | -/- | -/- | GG |
| *P69* | CA | CC | -/- | -/- | -/- | GG |
| *P71* | CC | CC | -/- | -/- | -/- | GG |
| *P84* | CA | CC | -/- | **del/-** | -/- | GG |
| *P85* | CA | CC | -/- | -/- | -/- | GG |
| *P95* | CC | CC | -/- | -/- | -/- | GG |
| *P96* | CC | CC | -/- | -/- | **delA/-** | **GC** |
| *P97* | AA | CC | -/- | -/- | -/- | GG |
| *P102* | CC | CC | -/- | -/- | -/- | GG |
| *P117* | CA | CC | -/- | -/- | -/- | GG |
| *P120* | AA | CC | -/- | -/- | -/- | GG |
| *P122* | AA | CC | -/- | -/- | -/- | GG |
| *P133* | AA | CC | -/- | -/- | -/- | GG |
| *P143* | CA | CC | -/- | -/- | -/- | GG |

Table presents identified genotypes with minor allele in bold; -/- means no deletion or insertion was found.

*Clinical aspects*

Table 4 shows the list of patients carrying functionally relevant genetic alterations. Due to the very low frequency of alterations with the predicted pathogenic effects, we evaluated just associations of the rs757110 SNP in *ABCC8* with clinical data of the patients. None of the analyzed characteristics (with age, menopausal status, tumor size, presence of lymph node metastasis, grade, expression of hormonal receptors, ERBB2 and Ki-67, progression-free survival, and response to the neoadjuvant therapy) associated significantly with the carriage of rare alleles in this polymorphism (data not shown).

## Discussion

The present study shows that exome sequencing of both target genes is feasible and can be used for further studies on their relevance for prognosis and prediction of therapy outcome of cancer and eventually other serious diseases.

The role of ABCC8 and ABCD2 in human cancer is underexplored. Despite the earlier reports on their expression in human breast, colorectal and pancreatic carcinomas and associations of their intratumoral expression with clinical data of patients (Hlavata *et al.* 2012, Hlavac *et al.* 2013, Mohelnikova-Duchonova *et al.* 2013) further information and functional aspects are missing. However, the recently reported link between the ABCC8 overexpression in animal model of brain metastasis and blood-tumor barrier permeability together with the demonstrated anti-tumor potential of its inhibition by glyburide (Thompson *et al.* 2013) raise further interest. Evaluation of functional connections between genotype and phenotype of ABCC8 may become major pharmacogenetic tool for

stratification of patients for such therapy.

ABCC8 also known as sulfonylurea receptor (SUR1, Miki *et al.* 1999) belongs to the most often analyzed genes in both neonatal and maturity-onset of the young (MODY) forms of diabetes mellitus and previous studies suggested its potential use as pharmacogenetic marker for decision between therapy by oral sulfonylureas or insulin (Gloyn *et al*. 2004, Pearson *et al.* 2006). Its gene product forms together with the product of *KCNJ11* gene, the Kir6.2 subunit (OMIM: 600937), the ATP-dependent potassium channel playing a critical role in glucose homeostasis (Bennett *et al*. 2010).

The present study identified three alterations in ABCC8 with the *in silico* predicted functional relevance for its phenotype. Two alterations were newly discovered compared with the previously published results of whole exome sequencing of ABCC8 (Bonnefond *et al.* 2010, Johansson *et al*. 2012, Proverbio *et al.* 2013) and dbSNP database (http://www.ncbi.nlm.nih.gov/snp). The rs757110 polymorphism leading to the substitution of alanine at 1369 residue to serine inside the nucleotide binding domain (NBD2) is suspected to modify proper function of the ABCC8 transporter (Krugluger *et al*. 2000). Indeed, PolyPhen-2 used in the present study predicted damaging effects particularly of its truncated isoform with substitution of alanine at 197 residue to serine in NBD2.

However, in the stratified analysis of the patient set the rs757110 polymorphism did not significantly associate with any of the followed clinical characteristics of breast carcinoma patients. Despite this result, due to the small size of the patient set, the possibility of clinical relevance of the examined polymorphism cannot be ruled out.

Whole exome sequencing study of the *ABCD2* gene coding ALDRP (adrenoleukodystrophy-related protein) was not published so far and thus, the presently reported data is novel as well as the method for its assessment. Therefore, in contrary to *ABCC8*, the *ABCD2* analysis revealed 62 (85 % of total) novel alterations with the predicted functional effect in three of them.

ABCD2 is active in peroxisomal transport of very long fatty acids, saturated fatty acids, monounsaturated acids and polyunsaturated fatty acids (Hlavac and Soucek 2015). The reported overexpression of ABCD2 in white adipose tissue during adipogenesis (Liu *et al.* 2010) demonstrates the key role of ABCD2 in lipid metabolism and together with the current epidemiological evidence supporting the role of obesity as a major cancer risk factor (Park *et al.* 2011) implicates a potential importance of ABCD2 for cancer development and progression.

Interestingly, ABCD2 knock-down was reported to stimulate apoptosis in ovarian cancer cell line SKOV3 after cisplatin treatment *in vitro* suggesting a possible link between ABCD2 and platinum resistance (LaCroix *et al.* 2014).

The small sample size of the analyzed patient group may be considered a major limitation of this study. However, the study was designed as the first step to enable large-scale screening targeted either at alterations with the predicted functional effect or at assessment of overall impact of exome alterations for the disease burden.

In conclusion, the present study provides new methods for the testing of genetic variability of ABCC8 and ABCD2 transporters with implications for screening of genetic background of diabetes, impairment of lipid homeostasis, and potentially also further research of their link to cancer.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

This work was supported by the Czech Science Foundation (grant no. P303/12/G163), Internal Grant Agency of the Czech Ministry of Health (grant no. 14055-3), the National Sustainability Program I (NPU I) (grant no. LO1503) provided by the Ministry of Education Youth and Sports of the Czech Republic, and by the MH CZ - DRO (National Institute of Public Health – NIPH, 75010330) program.

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# Příloha č. 2:

**Hlaváč V** et Souček P

**Role of family D ATP-binding cassette transporters (ABCD) in cancer**

Biochem. Soc. Trans., review. 2015; 43, 937–942 [IF: 3,194] 50 % *)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

# Role of family D ATP-binding cassette transporters (ABCD) in cancer

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## Abstract

**ATP-binding cassette (ABC) transporters, belonging to the family D, are expressed in peroxisomes, endoplasmic reticulum or lysosomes. ABCD transporters play a role in transport of lipids, bile acids and vitamin B12 and associate with peroxisomal disorders. ABCD1 performs transport of coenzyme A esters of very-long-chain fatty acids (VLCFA) in peroxisomes and a number of mutations in *ABCD1* gene were linked to an X-linked adrenoleucodystrophy (X-ALD). The role of ABCD transporters in tumour growth has not been studied in detail, but there is some evidence that ABCDs levels differ between undifferentiated stem or tumour cells and differentiated cells suggesting a possible link to tumorigenesis. In this mini-review, we discuss the available information about the role of ABCD transporters in cancer.**

## Introduction

ATP-binding cassette (ABC) transporters belong to membrane transporter proteins. Along with the other type of membrane transporters, the solute carrier transporters (SLC), they translocate chemical compounds across cellular and intracellular membranes.

ABC transporters form a superfamily of seven families A, B, C, D, E, F and G [1]. However; from the total number 48 human ABC transporters, up to 16 have been shown to transport chemotherapeutic drugs [2,3]. The expression pattern of ABC transporters is tissue-, cell type- and compartment-specific with large inter-individual variability [4–6]. [www.genecards.org].

ABCs transport most of the clinically and toxicologically relevant compounds, e.g., anti-cancer, anti-HIV, anti-depressant, antibiotic, anti-epileptic and analgesic drugs. Strong evidence suggests that ABCB1 [multi-drug resistance 1 (MDR1), Online Mendelian Inheritance in Man (OMIM): 171050], ABCC1 [MDR-associated protein 1 (MRP1), 158343] and ABCG2 (breast cancer resistance protein, BCRP, 603756) take part in MDR and modify chemotherapy response. The rest of members of the ABCC family coding MRP are also well characterized. For the remaining ABC transporters, scarce information about their role in cancer onset and progression exists. In this mini-review, we address current data indicating potential involvement of ABCD transporters in tumorigenesis, cancer progression and modulation of cancer treatment response.

**Key words:** ATP-binding cassette (ABC) transporters, cancer, differentiation, peroxisomes, progression.

**Abbreviations:** ABC, ATP-binding cassette; ALD, adrenoleucodystrophy; KRAS, Kirsten rat sarcoma oncogene homolog; MDR, multi-drug resistance; MRP, MDR-associated protein; PMP70, the 70-kDa peroxisomal membrane protein; VLCFA, very-long-chain fatty acids; X-ALD, X-linked adrenoleucodystrophy.

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## Transporters of the ABCD family

Peroxisomes are sub-cellular organelles present in virtually all eukaryotic cells. They perform a broad range of functions, e.g., $\beta$-oxidation of fatty acids. Peroxisomes are surrounded by a single membrane of similar composition to the endoplasmic reticulum [7]. ABCD, or also called adrenoleucodystrophy (ALD), transporter family in humans consists of four members: ALD protein (ALDP) coded by ABCD1 gene (300371), ALD-related protein (ALDRP) coded by ABCD2 (601081), the 70-kDa peroxisomal membrane protein (PMP70) coded by ABCD3 (170995) and P70R (or PMP69) coded by ABCD4 (603214) [1]. Chromosomal position, gene size, numbers of coding exons and amino acids and subcellular localization of human ABCD transporters are summarized by Kemp et al. [8].

All four mammalian members of ABCD family are half transporters and contain one of each transmembrane (TM) and nt-binding domains (NBDs). Functional ABCD assembles as either homodimer or heterodimer. ABCD1–3 transporters facilitate the transport of fatty acids from cellular matrix to peroxisomes (for hypothesized structure and overview of ABCDs substrate specificity see [8]).

It seems evident that lipid metabolism plays a role in cancer progression [9]. The number of peroxisomes depends on the degree of differentiation [10]. Therefore, the role of peroxisomal ABCD lipid transporters in cancer development, progression and therapy outcome is emerging and deserves detailed study.

## *ABCD1* (ALDP)

ABCD1 [11] is involved in transport of coenzyme A esters of saturated or monounsaturated very-long-chain fatty acids (VLCFA) with tails longer than 22 carbons (mostly C24:0 and C26:0) [12] (Table 1).

Mutations in *ABCD1* gene are linked to X-linked ALD (X-ALD, 300100), a progressive neurodegenerative peroxisomal


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**Table 1** | **Substrate specificities of ABCD transporters**

Abbreviations: DHCA, 3$\alpha$, 7$\alpha$-dihydroxy-5$\beta$-cholestanoyl-CoA; ER, endoplasmic reticulum; LCFA, long chain fatty acids; THCA, 3$\alpha$, 7$\alpha$, 12$\alpha$-trihydroxy-5$\beta$-cholestanoyl-CoA.

| Transporter | Subcellular localization | Substrate | Reference(s) |
|---|---|---|---|
| ABCD1/ABCD2 | Peroxisomal membrane | C24:0-CoA | [28,29] |
| | | C26:0-CoA | [28,29] |
| | | C18:1-CoA | [12,28] |
| | | C20:1-CoA | [27] |
| | | C22:1-CoA | [27,31] |
| | | C20:0-CoA | [27] |
| | | C22:0-CoA | [28,41] |
| ABCD2 | Peroxisomal membrane | C22:6$\omega$3-CoA | [27–29] |
| | | C24:6$\omega$3-CoA | [27,28] |
| | | C22:5$\omega$6 | [27] |
| | | C16:0-CoA | [41] |
| | | C18:0-CoA | [41] |
| ABCD3 | Peroxisomal membrane | LCFA (C20:5-CoA) | [41] |
| | | THCA-CoA | [42] |
| | | DHCA-CoA | [42] |
| | | Pristanoyl-CoA | [41,42] |
| | | Phytanoyl-CoA | [42] |
| | | Dicarboxylic acids | [41] |
| ABCD4 | ER membrane | unknown | [50] |
| | Lysosomal membrane | B12 | [51,52] |

disorder characterized by accumulation of VLCFA affecting lipid storage in brain and adrenal cortex [13,14].

Northern blot analysis of ABCD transporters in mouse brain showed highest ABCD1 expression in embryos and its gradual decrease during maturation from newborn to older mice [15] suggesting a possible link of ABCD1 to cell differentiation with parallel to tumorigenesis. However, the finding of 13-times higher ABCD1 transcript level in human differentiated macrophages compared with less differentiated primary monocytes points to a tissue- and cell type-specific role of ABCDs in cell differentiation [16].

In a comprehensive study, Szakacs et al. [17], assessed gene expression of all human ABC transporters in a large panel of different tumour cell lines. ABCD1 was found in a cluster of ABCs expressed specifically in melanoma cell lines. An inverse correlation between ABCD1 and ABCB1 gene expression implicated a possible link of ABCD1 to melanoma drug resistance [17]. Later study observed lower ABCD1 transcript expression levels in melanoma cell lines than in normal human epidermal melanocytes [18].

ABCD1 transcript (measured by real-time PCR) and protein (by immunohistochemistry in paraffin sections) was down-regulated in renal cell carcinomas compared with paired normal renal cell tissue samples ($n = 77$) [19]. In recent studies, we have measured the transcript levels of all human ABC transporters in breast, colorectal and pancreatic tumours and paired adjacent non-neoplastic tissues by real-time PCR. ABCD1 transcript levels were significantly overexpressed in breast carcinomas compared with non-neoplastic control tissues [20]. On the opposite, ABCD1 transcript levels in colorectal and pancreatic tumours compared with paired non-neoplastic control tissues were unaffected by tumorigenesis [21,22]. Nevertheless, pancreatic tumours with angioinvasion had higher levels of ABCD1 transcript when compared with the tumours without angioinvasion, suggesting its potential role in cancer spread [22]. Interestingly, of all human ABCs, just ABCD1 was overexpressed in near-normal human pancreatic ductal epithelial cells harbouring Kirsten rat sarcoma oncogene homolog (KRAS) G12V mutation under hypoxia compared with unaffected cells with KRAS G12V [23].

Investigation of $\beta$-oxidation impairment in primary fibroblasts from X-ALD and Zellweger syndrome patients revealed that ABCD1 dysfunction causes the defect in $\beta$-oxidation. This study also addressed transcript and protein levels of ABCDs in patients' fibroblasts. Interestingly, the transcript level did not differ between healthy control and X-ALD or Zellweger patients (both ABCD1 and ABCD3; ABCD2 level was negligible), but the ABCD1 protein expression was reduced in X-ALD cases [24]. This observation suggests the lack of correlation between ABCD1 transcript and protein levels and implies that transcriptomic results need cautious interpretation.

## *ABCD2* (ALDRP)

ABCD2 [25] is highly similar to ABCD1 (63 % homology) [26] and has overlapping substrate specificities with ABCD1 towards saturated fatty acids and monounsaturated acids. Nevertheless, ABCD2 has a distinct substrate specificity towards shorter VLCFA (C20:0-CoA and C22:0-CoA) and polyunsaturated fatty acids (PUFAs, C22:6-CoA and C24:6-CoA) [27–29]. Substrate specificities of ABCD2 and other members of ALD family are summarized in Table 1.

ABCD2 transcript was down-regulated during mesenchymal development (higher level in pluripotent embryonic stem cells compared with multipotent mesenchymal stem cells) [30]. ABCD2 protein is highly expressed in white adipose tissue and especially in cultured 3T3-L1 adipocytes and its expression increases during adipogenesis [31]. The observed rapid obesity onset, hepatic steatosis and insulin resistance in Abcd2-deficient mice fed with dietary erucic acids (C22:1$\omega$9) underlines the key role of ABCD2 in lipid metabolism [32]. Interestingly, the number of peroxisomes increased during differentiation of 3T3-L1 adipocytes and Abcd2 identifies a subclass of peroxisomes that may be specific for adipocytes [10]. Adipose tissue is a master regulator of energy balance and nutritional homoeostasis [33]

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and current epidemiological evidence supports the role of obesity as a major cancer risk factor [34].

ABCD2 knockdown led to increased apoptosis in ovarian cancer cell line ovarian adenocarcinoma (SKOV3) after cisplatin treatment *in vitro*. Furthermore, the ABCD2 gene expression was decreased by *miR-30d in vitro* suggesting a possible role of ABCD2 and potentially *miR-30d* in conferring platinum resistance [35].

ABCD2 levels were significantly down-regulated in breast tumours compared with non-neoplastic tissues of pre-treatment patients and of patients after neoadjuvant chemotherapy (post-treatment patients). In addition, higher ABCD2 transcript levels significantly associated with better response to neoadjuvant chemotherapy in post-treatment patients. However, protein levels assessed by immunoblotting in tumour tissues did not correlate with the gene transcript levels indicating that functional relevance needs further delineation [20]. Post-treatment patients with high transcript levels of ABCD2 in tumours (median cut-off) had significantly longer progression-free survival (log rank, $P = 0.005$) than patients with low levels ($n = 63$) [Hlaváč, V., Brynychová, V., Václavíková, R., Ehrlichová, M., Vrána, D., Pecha, V., Koževnikovová, R., Trnková, M., Gatěk, J., Kopperová, D., Gut, I. and Souček, P., unpublished results]. Alike breast cancer, we observed down-regulation of ABCD2 in tumours compared with paired non-neoplastic mucosa tissues in colorectal carcinoma [21], but levels in pancreatic carcinoma were unchanged [22]. From this point of view it is interesting that direct regulation of ABCD2 by $\beta$-catenin (cadherin-associated protein beta 1, CTNNB1, 116806) and transcription factor 4 (TCF4, 602272) was recently shown *in vitro* [36]. These members of Wnt signalling pathway were implicated in colorectal and pancreatic tumorigenesis [37,38].

## *ABCD3* (PMP70)

ABCD3 is the first peroxisomal ABC transporter identified [39]. The protein PMP70 has capacity to transport large spectrum of fatty acids. Although ABCD1, ABCD2 and ABCD3 have overlapping substrate specificities, ABCD3 has a major role in transport of long-chain unsaturated, long branched-chain and long-chain dicarboxylic fatty acids [40,41] (Table 1). Recently, *ABCD3* deletion in patient with hepatosplenomegaly has been found [42].

ABCD3 transcript was overexpressed almost eight times in human differentiated macrophages compared with primary monocytes from healthy donors [16].

Amplification of *ABCD3* (and *ABCD4*) measured by comparative genome hybridization (CGH) in several drug-resistant cell lines compared with drug-sensitive parental cell lines was observed implicating role of these genes in acquired chemoresistance [43].

ABCD3 protein and transcript are expressed in glioma tumour cells. The malignancy differentiation (grade) correlated with the gene and protein expression of ABCD3 and other peroxisomal genes. The transcript level was significantly higher in grade III or grade IV tumours than in grade II tumours and the protein level increased gradually with the grade. The level of triglycerides increased as well suggesting together that peroxisomes are strongly involved in glioma biology and correlate with malignancy progression [44]. ABCD3 transcript level measured by real-time PCR was higher in Y79 retinoblastoma cells than in the (commercially available) RNA pooled from different tissues [45].

Lauer et al. [46] found reduction in peroxisomes in colon cancer. In their study, ABCD3 was down-regulated in colorectal cancer tumours compared with unaffected colonic mucosa by immunoblotting and immunohistochemistry. However, the significant reduction in the PMP70 protein did not correlate with reduction in the corresponding mRNA (addressed by northern blot) [46]. Congruently with these results, our group has observed down-regulation of ABCD3 transcript levels in colorectal and pancreatic tumour tissues [21,22]. In contrast, transcript levels of ABCD3 were overexpressed in breast tumours [20] and no significant difference in ABCD3 transcript levels between samples from human skin and malignant melanoma patients was found as well [6].

ABCD3 and Ras-related nuclear protein GTPase-activating protein 1 (RanGAP1) have previously been identified among genes linked with prostate cancer using cDNA microarrays and gene–gene interaction studies revealed associations of SNPs surrounding ABCD3 with RanGAP1 expression [47]. ABCD3 transcript was 2-fold-increased in the metastatic prostate cancer cell line compared with normal prostate cells. Human prostate adenocarcinoma derived from bone metastasis (MDA-2PC-2B) metastatic cell line, derived from African American patient, showed the highest (10-fold) increase [47]. ABCD3 protein expression increased with increasing aggressiveness (Gleason score), age and pathology grade in Caucasian prostate cancer patients. However, ABCD3 was overexpressed to the same degree in both low and high Gleason score tumours in the African American patients [48]. Thus, the role of ABCD3 so far seems associated with prostate, glioma, breast and gastrointestinal cancer development.

## *ABCD4 (P70R)*

ABCD4 [26,49] shares 46 % sequence homology with human ABCD1. However, Kashiwayama et al. [50] reported that ABCD4 localizes to endoplasmic reticulum due to its hydrophobic N-terminal region. Recently, ABCD4 was shown to export vitamin B12 from lysosomes in human fibroblasts [51,52] and simultaneously was ABCD4 identified among 13 target loci associated with serum B12 and folate levels [53]. Association of ABCD4 with B12/folate pathway may have implications for colorectal cancer risk [54] and efficacy of therapy [55].

ABCD4 transcript was overexpressed in pluripotent embryonic stem cells compared with multipotent mesenchymal stem cells [30] and more than three times higher in human differentiated macrophages compared with primary monocytes [16].

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**Table 2 | Differences in expression of ABCD transporters in cancer and control tissues and clinically relevant associations**
Abbreviations: NACT, neoadjuvant chemotherapy; PFS, progression-free survival.

| | | Reference(s) | | Reference(s) | | Reference(s) |
|---|---|---|---|---|---|---|
| ABCD1 | Expression in tumour compared with non-neoplastic tissue | | Angioinvasive compared with without angioinvasion | | Drug resistant compared with drug sensitive | |
| Breast cancer | Up-regulation | [20] | – | | – | |
| Colorectal cancer | Unchanged | [21] | – | | – | |
| Pancreatic cancer | Unchanged | [22] | Up-regulation | [22] | – | |
| Melanoma (nine cell lines) | Down-regulation | [18] | – | | – | |
| Renal cell cancer | Down-regulation | [19] | – | | – | |
| ABCD2 | Expression in tumour compared with non-neoplastic tissue | | Response to NACT compared with non-response | | Drug resistant compared with drug sensitive | |
| Breast cancer | Down-regulation | [20] | Up-regulation | [20] | – | |
| Colorectal cancer | Down-regulation | [21] | – | | – | |
| Pancreatic cancer | Unchanged | [22] | – | | – | |
| Ovarian cancer (SKOV3) | – | | – | | Up-regulation | [35] |
| ABCD3 | Expression in tumour compared with non-neoplastic tissue | | High grade compared with low grade | | Drug resistant compared with drug sensitive | |
| Breast cancer | Up-regulation | [20] | – | | – | |
| Colorectal cancer | Down-regulation | [21,45] | – | | – | |
| Pancreatic cancer | Down-regulation | [22] | – | | – | |
| Melanoma | Unchanged | [6] | – | | – | |
| Retinoblastoma (Y79) | Up-regulation | [45] | – | | – | |
| Glioma | – | | Up-regulation | [44] | – | |
| Prostate cancer | – | | Up-regulation | [48] | – | |
| Neoplasms (23 cell lines) | – | | – | | DNA amplification | [43] |
| ABCD4 | Expression in tumour compared with non-neoplastic tissue | | Longer PFS compared with shorter PFS | | Drug resistant compared with drug sensitive | |
| Breast cancer | Down-regulation | [20] | – | | – | |
| Colorectal cancer | Down-regulation | [21] | Up-regulation | [21] | – | |
| Pancreatic cancer | Up-regulation | [22] | – | | – | |

ABCD4 transcript expression was significantly down-regulated in colorectal tumours compared with non-neoplastic mucosa tissues and the disease-free interval of patients treated by 5-fluorouracil-based adjuvant chemotherapy was significantly shorter in patients with low intratumoural transcript levels [21]. Accordingly, we have observed down-regulation of ABCD4 transcript levels in tumours compared with non-neoplastic tissues also in breast carcinoma patients [20]. In the opposite, ABCD4 transcript levels were significantly higher in pancreatic tumours compared with non-neoplastic tissues [22] suggesting its tissue-specific deregulation during tumorigenesis (Table 2).

## Conclusions

Taken together, the role of VLCFA accumulation in X-ALD and its proposed role in tumorigenesis is an interesting topic for further investigations. The observed down-regulation of ABCDs in certain tumour types may in some instances cause lipid accumulation and could then promote tumour growth and progression in a similar way as in X-ALD, i.e. by oxidative stress and stimulation of inflammation with subsequent loss of peroxisomal functions. In addition, tumours need lipids as construction material for their increased proliferation. One can speculate that the intracellular balance between lipid entities modulated by

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ABCDs is most probably more important than impairment of single transporter.

Results of ABCDs profiling of major human solid tumours (breast, colorectal and pancreatic carcinoma) suggest a cancer-specific deregulation of ABCDs and its potential as a therapy target, especially in breast carcinoma. The reported variations in expression between differentiated and less differentiated (pluri-/multi-potent) stem cells are most probably tissue-specific as well and may influence the potential of cancer stem cells to accelerate metastasis.

The revealed lack of correlation between transcript and protein levels of ABCD transporters needs a particular attention because it limits interpretation of existing transcriptomic data. In contrary to the availability of transcriptomic profiles in different tissue and cell types, a scarcity of protein data, especially information about post-translational regulation of ABCD family and its detailed structural topology precludes translation of current knowledge about ABCD transporters into clinical application in oncology.

## Funding

This work was supported by the Internal Grant Agency of Czech Republic [grant number IGA-NT13679-4]; the Czech Science Foundation [grant number GACR P303/12/G163]; and by the Ministry of Health, Czech Republic DRO program [grant number 75010330].

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Received 12 May 2015
doi:10.1042/BST20150114

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# Příloha č. 3:

**Hlaváč V,** Brynychová V, Václavíková R, Ehrlichová M, Vrána D, Pecha V, Trnková M, Kodet R, Mrhalová M, Kubáčková K, Gatěk J, Vážan P, Souček P

**The role of cytochromes P450 and aldo-keto reductases in prognosis of breast carcinoma patients**

Medicine (Baltimore). 2014;93(28):e255 [IF: 4,867] 40 % *)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

OPEN

# The Role of Cytochromes P450 and Aldo-Keto Reductases in Prognosis of Breast Carcinoma Patients

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**Abstract:** Metabolism of anticancer drugs affects their antitumor effects. This study has investigated the associations of gene expression of enzymes metabolizing anticancer drugs with therapy response and survival of breast carcinoma patients.

Gene expression of 13 aldo-keto reductases (AKRs), carbonyl reductase 1, and 10 cytochromes P450 (CYPs) was assessed using quantitative real-time polymerase chain reaction in tumors and paired adjacent nonneoplastic tissues from 68 posttreatment breast carcinoma patients. Eleven candidate genes were then evaluated in an independent series of 50 pretreatment patients. Protein expression of the most significant genes was confirmed by immunoblotting.

AKR1A1 was significantly overexpressed and AKR1C1–4, KCNAB1, CYP2C19, CYP3A4, and CYP3A5 downregulated in tumors compared with control nonneoplastic tissues after correction for multiple testing. Significant association of CYP2B6 transcript levels in tumors with expression of hormonal receptors was found in the posttreatment set and replicated in the pretreatment set of patients. Significantly higher intratumoral levels of AKR1C1, AKR1C2, or CYP2W1 were found in responders to neoadjuvant chemotherapy compared with nonresponders. Patients with high AKR7A3 or CYP2B6 levels in the pretreatment set had significantly longer disease-free survival than patients with low levels. Protein products of AKR1C1, AKR1C2, AKR7A3, CYP3A4, and carbonyl reductase (CBR1) were found in tumors and those of AKR1C1, AKR7A3, and CBR1 correlated with their transcript levels. Small interfering RNA-directed knockdown of AKR1C2 or vector-mediated upregulation of CYP3A4 in MDA-MB-231 model cell line had no effect on cell proliferation after paclitaxel treatment in vitro.

Prognostic and predictive roles of drug-metabolizing enzymes strikingly differ between posttreatment and pretreatment breast carcinoma patients. Mechanisms of action of AKR1C2, AKR7A3, CYP2B6, CYP3A4, and CBR1 should continue to be further followed in breast carcinoma patients and models.

(*Medicine* 93(28):e255)

**Abbreviations**: ADR = adriamycin, AKR = aldo-keto reductase, CBR = carbonyl reductase, CI = confidence interval, CYP = cytochrome P450, DFS = disease-free survival, ER = estrogen receptor, ERBB2 = V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2, FACFDR = false discovery rate, GAPDH = glyceraldehyde phosphate dehydrogenase, HR = hazard ratio, KCNAB = shaker-related voltage-gated potassium channel, subfamily beta, Ki67 = proliferation-related Ki-67 antigen, NACT = neoadjuvant chemotherapy, NS = not significant, p53 = tumor protein p53, PCT = paclitaxel, pN = pathological lymph node involvement, PR = progesterone receptor, pT = pathological tumor size, qPCR = quantitative real-time polymerase chain reaction, REF = reference gene, REST = Relative Expression Software Tool, RIN = RNA integrity number, TRG = target gene.

Editor: Yong Liu.
Received: May 14, 2014; revised: October 2, 2014; accepted: October 6, 2014.
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This work was supported by the grants of the Czech Science Foundation No. 13-25222J and the Internal Grant Agency of the Czech Ministry of Health No. NT/14055-3.
VH, VB, and ME carried out the experimental studies, participated in the evaluation of results, and drafted the manuscript. PS and RV designed and coordinated the study, performed statistical analyses, and drafted the manuscript. DV, VP, JG, and KK recruited patients, collected clinical data, and drafted the manuscript. MT, MM, RK, and PV performed preparation and pathological assessment of the tissue specimens and drafted the manuscript. All authors read and approved the final manuscript.
The authors have no conflicts of interest to disclose.
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ISSN: 0025-7974
DOI: 10.1097/MD.0000000000000255

## INTRODUCTION

Breast carcinoma is the most common cancer in women worldwide.[1] The prognosis of breast carcinoma patients vastly depends on the response of the tumor cells to chemotherapy. Decreased uptake or eventually increased efflux of drugs, increased DNA repair or reduced apoptosis, and inactivation of anticancer drugs by biotransformation enzymes may contribute to the development of multidrug resistance.[2]

Phase I biotransformations typically involve substrate oxidation by the cytochrome P450 (CYP) monooxygenases. About 20 enzymes from 57 known CYPs are active in metabolism of procarcinogens and drugs. Most of them lack important functional polymorphisms, but *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2D6* are highly polymorphic suggesting a potential effect on their expression.[3]

Cyclophosphamide is a prodrug that must undergo activation by CYP2C9, CYP2C19, CYP3A4, and CYP3A5.[4,5] CYP2B6 also participates in cyclophosphamide activation in the liver, but its role in the response to cyclophosphamide in cancer patients has not been proven yet.[6] CYP2C8, CYP3A4, and CYP3A5 are major taxane-metabolizing enzymes.[7,8] Roles

of CYP1A2, CYP2A6, and CYP2C8 in 5-fluorouracil formation from a prodrug tegafur have been described as well.[9] *CYP2C19* and *CYP2D6* polymorphisms have recently been associated with therapeutic outcome of tamoxifen-treated breast carcinoma patients.[10]

CYP2C9, CYP2D6, and CYP3A4 mRNA expression has unambiguously been detected in mammary gland.[11,12] Strong protein expression of CYP2S1 and CYP3A4 has been associated with shorter survival time of breast carcinoma patients.[13] Despite the knowledge about CYP2W1 substrate specificity is limited,[14] its overexpression in colorectal carcinomas[15] raises interest about future plans for CYP2W1-based cancer therapy.[6]

Carbonyl reductases (CBRs) and aldo-keto reductases [AKR and Voltage-gated K+ channel beta subunit (KCNAB)] are involved in redox transformations of broad spectrum of carbonyl group-containing xenobiotics, for example, in the transformation of adriamycin to its inactive metabolite adriamycinol.[16–18] Mammalian AKRs are divided into 3 families AKR1, KCNAB, and AKR7 with 13 identified AKR proteins: AKR1A1 (aldehyde reductase), AKR1B1 and AKR1B10 (aldose reductases), AKR1C1, AKR1C2, AKR1C3, and AKR1C4 (hydroxysteroid dehydrogenases), AKR1D1 (Δ4–3-ketosteroid-5-β-reductase), KCNAB1, KCNAB2, and KCNAB3 (voltage-gated potassium channels), and AKR7A2 and AKR7A3 (aflatoxin reductases).[16]

Taken together, available data in the literature suggest a potential role of drug-metabolizing enzymes in the response of patients to anticancer therapy. However, studies in target tissues of patients are limited and therefore urgently needed for translation of functional data into clinical practice. A comprehensive set of metabolizing enzymes involved in the chemotherapy outcome is, thus, still to be defined.

This study explored gene expression levels of drug-metabolizing enzymes in the posttreatment tissues from breast carcinoma patients treated by neoadjuvant chemotherapy (NACT). Expression profiles were compared with clinical data and with response of the patients to NACT in order to identify putative biomarkers with prognostic and predictive value. Two cohorts of pretreatment patients were then used for comparison and assessment of biological relevance of putative biomarkers on the protein level.

## METHODS

### Materials

Phenol, chloroform, RNase A, proteinase K, ultrapure agarose, and other general chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic). Deoxynucleotides for polymerase chain reaction (PCR) and molecular weight standard for electrophoresis (ΦX174DNA/HaeIII digest) were products of New England Biolabs, Inc (Ipswich, MA). TaqPurple DNA polymerase and Combi PPP Master Mix for PCR were supplied by Top-Bio s r.o. (Prague). Protein standards for immunoblotting were kindly provided by Prof Paul F. Hollenberg, University of Michigan, Ann Arbor, MI (P450 2B6) and Prof F. Peter Guengerich, Vanderbilt University, Nashville, TN (P450 3A4).

### Patients

Posttreatment tissue samples of human carcinomas of the mammary gland were prospectively obtained from 68 incident breast carcinoma patients diagnosed at the Department of Oncosurgery, Medicon, Prague, during 2006–2010. Patients were treated by NACT based on 5-fluorouracil/adriamycin/cyclophosphamide or 5-fluorouracil/epirubicin/cyclophosphamide and eventually taxanes (for NACT regimens see Table, Supplemental Digital Content 1, http://links.lww.com/MD/A124). Paired adjacent tissue samples without morphological signs of carcinoma (nontumor controls) were available from 43 patients. Collection and pathological processing of tissue samples and retrieval of data was performed as described before.[19]

Pretreatment tissue samples of human carcinomas of the mammary gland were prospectively obtained from 50 incident breast carcinoma patients diagnosed at the Faculty Hospital in Motol, Prague, during 2003–2007. Paired adjacent tissue samples without morphological signs of carcinoma (nontumor controls) were available from 31 patients. Patients were treated by adjuvant chemotherapy and eventually hormonal therapy after surgery (Table, Supplemental Digital Content 1, http://links.lww.com/MD/A124). Collection and pathological processing of tissue samples and retrieval of data was done as described before[20] (Text, Supplemental Digital Content 2, http://links.lww.com/MD/A124).

For analysis of protein levels of candidate genes, third set was established. Pretreatment tumor tissue samples of human carcinomas of the mammary gland were prospectively obtained from 42 incident histologically verified breast carcinoma patients diagnosed at the Department of Surgery, Hospital Atlas, Zlin, during 2012. Collection and handling of tissue samples and clinical data retrieval adhered to the above-described design (for study flow diagram, see Figure, Supplemental Digital Content 3, http://links.lww.com/MD/A124).

The following data on patients were retrieved from medical records: age, menopausal status, date of diagnosis, personal and family history of cancer, tumor size (pT), lymph node (pN) and distant metastasis (cM), clinical stage, histological type and grade of tumor, expression of estrogen receptor (ER), progesterone receptor (PR), V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2), p53 protein, and Ki-67 protein (for all clinical data, see Table, Supplemental Digital Content 4, http://links.lww.com/MD/A124).

All patients after the primary chemotherapy and surgery were followed for local or distant relapse. Response to NACT was evaluated by Response Evaluation Criteria in Solid Tumors, as described.[21]

All patients were asked to read and sign an informed consent. The study was approved by the Ethical Commission of the National Institute of Public Health in Prague.

### Isolation of Total RNA and cDNA Preparation

Total RNA was isolated from snap frozen tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA quantity and quality (RIN) was assessed and complementary DNA (cDNA) was synthesized using 0.5 μg of total RNA as described before.[20] The cDNA was then preamplified using 25 μL of TaqMan PreAmp Master Mix and a pool of 24 specific TaqMan Gene Expression Assays (Life Technologies Corp, Carlsbad; listed in Table, Supplemental Digital Content 5, http://links.lww.com/MD/A124) according to the published procedure.[19]

### Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was done and results were evaluated as described before.[19] Samples from the posttreatment set were preamplified using TaqMan PreAmp Master Mix (Life Technologies Corp). cDNA from the pretreatment set


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was used for quantification directly without preamplification procedure.

The relative standard curve was generated from 5 log dilutions of 1 nontumor tissue sample (calibrator). Amplification efficiencies for each reference gene (REF) and target gene (TRG) were calculated applying the formula efficiency $= 10^{-1/\text{slope}} - 1$.

*EIF2B1*, *MRPL19*, *IPO8*, and *UBB* were selected as the most stable reference genes for data normalization (Text, Supplemental Digital Content 2, http://links.lww.com/MD/A124). The qPCR study design adhered to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines.[22]

Gene expression and clinical data of the evaluation set were submitted to Gene Expression Omnibus repository under accession number GSE56259.

## Immunoblotting in Human Breast Carcinoma Tissues

Tumor tissue samples ($n = 42$) were stored at $-80°C$ before protein isolation. Samples were grinded using a mortar and pestle and then protein and total RNA was isolated using Allprep DNA/RNA/Protein Mini kit (Qiagen; Hildesheim, Germany) according to the manufacturer's protocol. Total RNA was then used for qPCR of CYP3A4, CBR1, AKR1C1 (Hs04230636_sH), AKR1C2, and AKR7A3 as described above. Protein concentration was determined and immunoblotting was done as previously described.[19] Briefly, 20 μg of protein was used for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel) and transferred onto 0.2 μm Protran nitrocellulose membrane (Whatman; Kent, UK). Protein standards of CYP2B6, CYP2S1, and CYP3A4 were used in amount of 0.25–1 pmol of purified protein per lane. First, membranes were incubated in blocking solution (Clear Milk Blocking Buffer; Pierce Thermo Scientific, Rockford, IL). Then, membranes were incubated with primary antibodies against CYP2B6 (dilution 1:200; Abgent, San Diego, CA), CYP2S1 (dilution 1 μg IgG/mL), CYP3A4 (dilution 5 μg IgG/mL[23]), AKR1C1 (dilution 1:1000; Aviva Systems Biology, San Diego), AKR1C2 (dilution 1:100; Aviva Systems Biology), AKR7A3 (dilution 1:1000; Genetex, Inc, Irvine, CA), CBR1 (dilution 1:1000; Genetex), or glyceraldehyde phosphate dehydrogenase (GAPDH) (dilution 1:1000; Cell Signaling Technology, Danvers, MA) overnight at 4°C. Membranes were then incubated 2 hours at room temperature with anti-rabbit horseradish-peroxidase-conjugated secondary antibodies (dilution 1:10,000; Sigma Aldrich, Prague, Czech Republic). Protein bands were visualized with an Enhanced Chemiluminescence Detection System (Pierce Biotechnology, Thermo Scientific Pierce Protein Research Products, Rockford, USA) by Carestream Gel Logic 4000 PRO Imaging System (Carestream Health, New Haven, CT). Densitometry was performed using Carestream v5.2 program (Carestream Health) as previously described.[19]

## Cells and Culture Conditions

Human breast carcinoma MDA-MB-231 cell line (without expression of hormonal receptors and ERBB2, ie, triple negative) was purchased from American Type Culture Collection (Manassas, VA). Cells between passages 4 and 40 were used for all experiments. Cell line was authenticated and genomic stability monitored in the fourth and 40th passages by short tandem repeat profiling using PowerPlex ESI 17 Pro System (Promega Corp, Madison, WI). Cells were cultured in basic medium with added 10% fetal bovine serum in a humidified atmosphere of 5% $CO_2$ at 37°C. RPMI 1640 containing extra L-glutamine (300 μg/mL), sodium pyruvate (110 μg/mL), 15 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid buffer, penicillin (100 U/mL), and streptomycin (100 μg/mL) was used as a basic medium. The cells were trypsinized by 0.25% trypsin and 0.02% Ethylenediaminetetraacetic acid in phosphate-buffered saline (all chemicals from PANBiotech GmbH, Aidenbach, Germany).

## AKR1C2 siRNA Knockdown

Cells were seeded at $5 \times 10^4$ per well (approximately at 70% confluence) of a 24-well plate in culture medium without antibiotics and cultured overnight. Next day culture medium was replaced by transfection mix. For knockdown, a sample of the pools of target small interfering RNA (siRNA) (predesigned, AKR1C2 siRNA, ID: s3991), or positive control (GAPDH siRNA, cat. no.: 4390849) or negative control (cat. no.: 4390846) (all 15 nM/well) in Reduced-Serum Minimal Essential Medium (OPTIMEM) was incubated with Lipofectamine 3000 reagent (all chemicals from Life Technologies), and added to OPTIMEM-conditioned cells in a total volume of 250 μL. After 24 hours incubation, an equal volume of culture medium without antibiotics with 20% fetal bovine serum was added, resulting in final concentrations of 10% fetal bovine serum (500 μL/well). Knockdown efficiencies were determined by qPCR and immunoblotting after 48 hours of growth after addition of culture medium.

## Transfection With pcDNA3.1–CYP3A4 Vector

Cells were seeded at $5 \times 10^4$ per well (approximately at 70% confluence) of the 24-well plate in culture medium and cultured overnight. Next day, culture medium was replaced by the fresh complete culture medium (500 μL) including 50 μL of DNA–lipid complex (0.5 μg of plasmid pcDNA 3.1–CYP3A4 or empty plasmid pcDNA 3.1 as negative control; GenScript, Piscataway, NJ). Cells were transfected by mixing with Lipofectamine 3000 reagent according to the instructions of manufacturer (Life Technologies). After 48 hours of transfection, cells were washed and supplemented with the fresh culture medium. Next day, cells were harvested and seeded at approximately 25% confluence onto 24-well plate in culture medium with various concentrations (100, 500, and 1000 μg/mL) of Geneticin (Santa Cruz Biotechnology, Dallas, TX) for selection of geneticin-resistant cells. Selective media were replenished every 3 days and percentage of surviving cells was monitored. After 9 days, 500 μg/mL of geneticin was selected to maintain cell line expressing CYP3A4. CYP3A4 expression was monitored by qPCR and immunoblotting.

## Cell Proliferation Assessment by Flow Cytometry

Cells were seeded at $1 \times 10^5$ per well of the 24-well plate and propagated in culture medium. Next day (after 18 hours), culture medium was replaced by the culture medium without drugs (control) or with 100 nM paclitaxel (PCT) or 30 μM adriamycin (LC Laboratories, Woburn, MA). Cells were harvested after 24 hours and fixed in 70% ethanol at 4°C overnight. Fixed cells were washed with phosphate-buffered saline, incubated with 40 μg/mL propidium iodide and 100 μg/mL RNase in phosphate-buffered saline, and cell cycle was analyzed using flow cytometer FACSVersa (Becton, Dickinson and Company, Franklin Lakes, NJ). CYP3A4 and AKR1C2 expression was monitored by qPCR and immunoblotting in parallel samples 48 hours after exposure to drugs.

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## Data Analysis

Raw cycle threshold (Ct) data were analyzed by Relative Expression Software Tool (REST) 2009 program (Qiagen, Hildesheim, Germany). Each sample was assayed in duplicate and the mean value was used for calculations. Samples with Ct >40 were treated as missing data. For statistical analyses of associations of transcript levels with clinical data nonparametric tests (Kruskal–Wallis, Mann–Whitney, and Spearman rank) were used. Tested variables were as follows: menopausal status (premenopausal vs postmenopausal), tumor size in millimeter and pT (pT1 vs pT2–4), lymph node metastasis (pN0 vs pN1–3), histological type (ductal vs other invasive breast carcinoma), pathological grade (G1 or G2 vs G3), stage (SI vs SII–SIII), ER, PR, ERBB2, and p53 expression (positive vs negative), Ki-67 expression in percentage of positive tumor cells; and response to NACT (partial pathological response vs stable disease or progression). Samples with complete pathological response after NACT have not been included into the study because of the lack of tumor tissue. Disease-free survival (DFS) was defined as the time elapsed between surgical treatment and disease progression or death from any cause.[20] Patients lost to follow-up (n = 5 in the posttreatment set) were excluded from the DFS analyses. DFS was evaluated by the Kaplan–Meier method and the log-rank test was used for evaluation of the compared groups of patients. For multivariate analysis, the Cox proportional hazards model was used. *P* values are departures from 2-sided tests. A *P* value of <0.05 was considered statistically significant. Statistical analyses were done using SPSS v16.0 program (SPSS Inc, Chicago, IL). The correction for false discovery rate (FDR) was applied according to Benjamini and Hochberg[24] and q-values are provided for each comparison.

**TABLE 1.** Differences in Transcript Levels Between Tumor and Control Tissues of Breast Carcinoma Patients

| Gene Symbol | *P* Value* | Tumor vs Control | Fold Change |
|---|---|---|---|
| **AKR1A1** | **<0.001‡** | ↑ | **1.55** |
| AKR1B1 | NS | — | 1.13 |
| AKR1B10 | 0.004 | ↑ | 3.02 |
| **AKR1C1** | **<0.001‡** | ↓ | **0.18** |
| **AKR1C2** | **<0.001‡** | ↓ | **0.14** |
| **AKR1C3** | **<0.001‡** | ↓ | **0.36** |
| **AKR1C4** | **<0.001‡** | ↓ | **0.09** |
| AKR1D1 | NS | — | 1.63 |
| AKR7A2 | NS | — | 1.09 |
| **AKR7A3** | **0.022** | ↑ | **1.62** |
| **CBR1** | **NS** | — | **0.95** |
| CYP1A2† | 0.029 | ↑ | 3.81 |
| **CYP2B6** | **0.027** | ↑ | **2.55** |
| **CYP2C8** | **NS** | — | **0.90** |
| CYP2C9 | NS | — | 0.70 |
| CYP2C19† | <0.001‡ | ↓ | 0.35 |
| CYP2D6† | 0.035 | ↑ | 1.75 |
| **CYP2S1†** | **0.007** | ↑ | **1.44** |
| CYP2W1 | 0.045 | ↑ | 2.46 |
| **CYP3A4** | **<0.001‡** | ↓ | **0.19** |
| **CYP3A5** | **<0.001‡** | ↓ | **0.27** |
| **KCNAB1** | **<0.001‡** | ↓ | **0.32** |
| KCNAB2 | 0.026 | ↑ | 1.32 |
| KCNAB3 | 0.012 | ↑ | 1.34 |

*Significantly deregulated genes by the REST2009 software (*P* values displayed). †n = 52 (n = 68 for the rest of genes). ‡Results, which passed correction for multiple testing (q = 0.002). AKR = aldo-keto reductase, CYP = cytochrome P450, NS, not significant. Results from the posttreatment set that have been confirmed in pretreatment set of patients are depicted in bold.

# RESULTS

## Transcript Levels in Tumors and Nonneoplastic Control Tissues

AKR1A1, AKR1B10, AKR7A3, KCNAB2, and KCNAB3 were significantly overexpressed in tumors compared with nonneoplastic control tissues from the posttreatment set. On the opposite, AKR1C1, AKR1C2, AKR1C3, AKR1C4, and KCNAB1 were significantly downregulated in tumors. CYP1A2, CYP2B6, CYP2D6, CYP2S1, and CYP2W1 were significantly overexpressed whereas CYP2C19, CYP3A4, and CYP3A5 were significantly downregulated in tumors. No significant changes in expression of AKR1B1, AKR1D1, AKR7A2, CBR1, CYP2C8, and CYP2C9 between tumor and control tissues were found. Fold change between tumor and control tissues (mean expression values) with *P* values calculated by REST 2009 are listed in Table 1.

## Associations of Transcript Levels With Clinical Data in the Posttreatment Set

Associations of transcript levels of all genes with clinical data were analyzed, but to retain concise style only significant results are reported in Table 2. For this purpose, solely gene expression levels in tumors were evaluated.

Postmenopausal patients had significantly higher AKR1B10 levels ($P = 0.026$) than the premenopausal patients. Tumor size negatively correlated with CYP2C8 and CYP2C19 levels (Table 2). Patients without lymph node metastasis had significantly higher intratumoral CYP2C9 levels than patients with lymph nodes involved ($P = 0.049$). CBR1 levels were significantly higher in grade 3 (undifferentiated) tumors than in grade 1 or 2 (well or moderately differentiated). On the opposite, AKR7A3, CYP2B6, and CYP2C9 levels were significantly higher in grade 1 or 2 tumors compared with grade 3 . CYP3A5 levels were significantly higher in tumors expressing ERBB2 than in ERBB2 negative. Conversely, AKR7A3, CYP2B6, and CYP2C8 levels were significantly higher in ER expressing tumors than in those without ER expression. AKR7A3 and CYP2B6 levels were significantly higher in PR expressing tumors than in those without PR expression. AKR1C4 and CBR1 levels positively, and AKR7A3 and CYP2B6 levels negatively, correlated with Ki-67 protein expression. When correction for multiple testing (Benjamini–Hochberg FDR) was applied, only associations between CYP2B6 levels and grade, and expression of ER, PR, and Ki-67 remained significant (Table 2).

Patients with partial response (responders, n = 38) to NACT had significantly higher intratumoral AKR1C1, AKR1C2, or CYP2W1 transcript levels than patients with stable or progressive disease, that is, nonresponders (n = 24) ($1.17 \pm 0.15$ vs. $1.29 \pm 0.13$, $P = 0.003$; $1.81 \pm 0.19$ vs $1.96 \pm 0.25$, $P = 0.016$; and $1.64 \pm 0.14$ vs $1.72 \pm 0.13$, $P = 0.025$; q = 0.004 for all; respectively). Three patients solely treated by hormonal regimens and 2 patients with unknown response were excluded from this analysis. Patients with intratumoral CYP3A4 or AKR1C2 levels higher than median had significantly longer DFS than the remaining patients (n = 63, mean DFS: 71.8 vs 61.5 months, $P = 0.015$, DFS:


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**TABLE 2.** Significant Associations of Intratumoral Transcript Levels With Clinical Data of Patients in the Posttreatment Set

| Gene | Tumor Size, mm† | Grade* | | ER Expression* | | PR Expression* | | Ki-67 Expression† |
|---|---|---|---|---|---|---|---|---|
| | | 1 or 2 | 3 | Positive | Negative | Positive | Negative | |
| AKR1C4 | NS | NS | | NS | | NS | | ρ = 0.325<br>P = 0.031 |
| AKR7A3 | NS | 1.09 ± 0.10<br>P = 0.028 | 1.14 ± 0.11 | **1.09 ± 0.11**<br>**P = 0.020** | **1.15 ± 0.09** | **1.09 ± 0.11**<br>**P = 0.032** | **1.15 ± 0.09** | ρ = −0.259<br>P = 0.034 |
| CBR1 | NS | 1.10 ± 0.05<br>P = 0.019 | 1.08 ± 0.05 | NS | | NS | | ρ = 0.241<br>P = 0.049 |
| CYP2B6 | NS | 1.22 ± 0.18<br>P < 0.001‡ | 1.51 ± 0.21 | **1.28 ± 0.21**<br>**P < 0.001‡** | **1.52 ± 0.22** | **1.30 ± 0.22**<br>**P = 0.002‡** | **1.50 ± 0.24** | ρ = −0.484<br>P < 0.001‡ |
| CYP2C8 | ρ = −0.348<br>P = 0.005 | NS | | **1.57 ± 0.13**<br>**P = 0.024** | **1.64 ± 0.08** | NS | | NS |
| CYP2C9 | NS | 1.41 ± 0.17<br>P = 0.022 | 1.48 ± 0.11 | NS | | NS | | NS |
| CYP2C19 | ρ = −0.284<br>P = 0.046 | NS | NS | | | NS | | NS |

For statistical analyses, a ratio of Ct for particular target gene (TRG) to arithmetic mean of Ct for all reference genes (TRG/REF) was calculated for each sample. Therefore, the lower is the TRG/REF ratio, the higher is the TRG transcript level. All clinical data and all genes have been analyzed, but to retain concise style only significant changes are reported. *P values were calculated by the Mann–Whitney test. Mean ± SD values are TRG/REF calculated by the ANOVA test to assess directions of associations. †P and ρ values were calculated by the Spearman rank test. ‡Results that passed correction for multiple testing (q = 0.004). AKR = aldo-keto reductase, CYP = cytochrome P450, ER = estrogen receptor, NS = not significant, PR = progesterone receptor, SD = standard deviation. Results confirmed in the pretreatment set are depicted in bold.

71.6 vs 60.9 months, $P = 0.012$, respectively; Figure 1). Multivariate analysis using the Cox regression hazards model with pT, pN, grade, and ER as covariates has confirmed these associations (hazard ratio [HR] = 8.79, 95% confidence interval [CI] = 1.09–70.56, and $P = 0.041$ for CYP3A4 and HR = 9.82, 95% CI = 1.02–94.05, $P = 0.048$ for AKR1C2).

## Associations of Transcript Levels With Clinical Data in the Pretreatment Set

Genes significantly associated with the most important clinical data as grade, expression of hormonal receptors, response to NACT or DFS, and genes strongly deregulated in tumors (significant after correction for multiple testing) in the posttreatment set were included into the study of pretreatment patients. Thus, AKR1A1, AKR1C1, AKR1C2, AKR1C3, AKR1C4, AKR7A3, KCNAB1, CBR1, CYP2B6, CYP2C8, CYP2S1, CYP3A4, and CYP3A5 were further followed in this patient set. As opposed to the posttreatment set no amplification of cDNA was used in the pretreatment set. CYP2C19 and CYP2W1 could not be validated in the pretreatment set owing to gene expression levels below the limit of quantification in samples without preamplification.

As for the posttreatment set, several associations were found (Table 3). However, after correction for multiple testing,

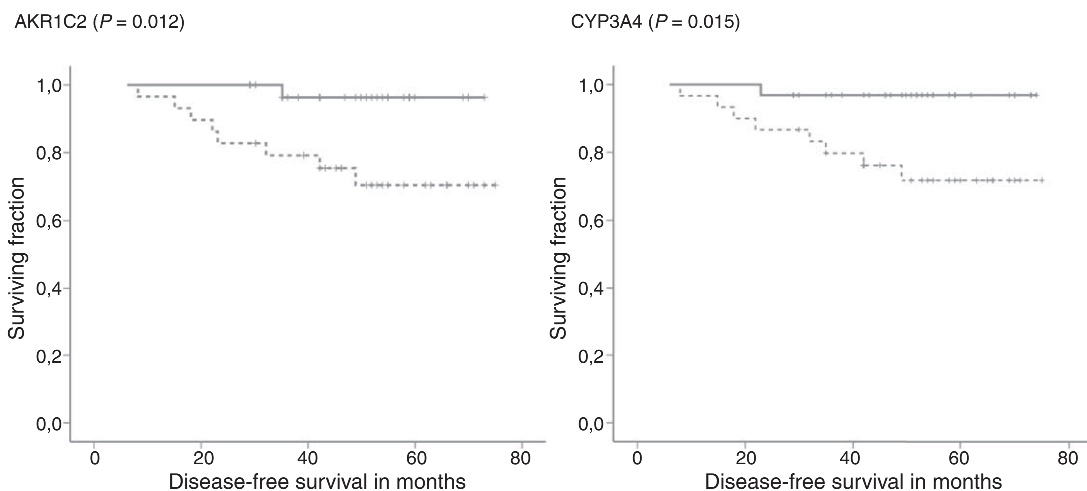


**FIGURE 1.** Associations between gene expression levels and DFS of posttreatment patients. Kaplan–Meier survival curves were plotted for patients (n = 65) divided into 2 groups according to the median of transcript levels in tumors. Dashed lines represent the group with lower transcript levels and solid lines represent the group with higher levels than median. Differences between groups were compared using log-rank test. Gene names and significant differences between groups are displayed. All clinical data have been analyzed, but to retain concise style only significant changes are reported. DFS = disease-free survival.

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**TABLE 3.** Significant Associations of Intratumoral Transcript Levels With Clinical Data of Patients in the Pretreatment Set

| Gene | Tumor Size†, mm | Grade* | | ER Expression* | | PR Expression* | | ERBB2* | | P53 Expression* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 or 2 | 3 | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Negative |
| AKR1C1 | $\rho = -0.333$<br>$P = 0.019$ | NS | | NS | | NS | | NS | | NS | |
| AKR1C3 | NS | NS | | NS | | NS | | $1.18 \pm 0.10$<br>$P = 0.045$ | $1.11 \pm 0.07$ | NS | |
| AKR7A3 | NS | NS | | **$1.00 \pm 0.11$**<br>**$P < 0.001$‡** | **$1.14 \pm 0.09$** | **$1.01 \pm 0.12$**<br>**$P = \pm 0.050$** | **$1.08 \pm 0.13$** | NS<br>NS | | $1.12 \pm 0.09$<br>$P = 0.006$ | $1.01 \pm 0.12$ |
| CYP2B6 | NS | NS | | **$0.98 \pm 0.09$**<br>**$P < 0.001$‡** | **$1.28 \pm 0.14$** | **$0.99 \pm 0.12$**<br>**$P = 0.001$‡** | **$1.18 \pm 0.19$** | NS | | $1.22 \pm 0.18$ | $1.01 \pm 0.13$<br>$P < 0.001$‡ |
| CYP2C8 | NS | NS | | **$1.44 \pm 0.08$**<br>**$P < 0.026$** | **$1.51 \pm 0.08$** | NS | | NS | | NS | |
| CYP2S1 | $\rho = -0.302$<br>$P = 0.039$ | NS | | NS | | NS | | $1.12 \pm 0.05$<br>$P = 0.046$ | $1.08 \pm 0.03$ | NS | |
| CYP3A4 | $\rho = -0.314$<br>$P = 0.030$ | NS | NS | NS | NS | | | | | NS | |

For statistical analyses, a ratio of Ct for a particular target gene (TRG) to arithmetic mean of Ct for all reference genes (TRG/REF) was calculated for each sample. Therefore, the lower is the TRG/REF ratio, the higher is the TRG transcript level. All clinical data have been analyzed, but to retain concise style only significant changes are reported. *$P$ values were calculated by the Mann–Whitney test. Mean ± SD values are TRG/REF calculated by the ANOVA test to assess directions of associations. †$P$ values and ρ values were calculated by the Spearman rank test. ‡Results that passed correction for multiple testing (q = 0.004). Confirmed results from the posttreatment set are depicted in bold. AKR = aldo-keto reductase, ANOVA = analysis of variance, CYP = cytochrome P450, ER = estrogen receptor, ERBB = V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2, NS, not significant, PR = progesterone receptor, SD = standard deviation.

only associations between AKR7A3 and expression of ER and those between CYP2B6 and expression of PR, ER, and p53 remained significant. Association between CYP2B6 and expression of hormonal receptors has also previously been observed in the posttreatment set (Table 2). Associations between AKR7A3 or CYP2C8 and expression of ER were also observed in both sets although they did not pass correction for multiple testing in one or both sets. Notable associations between AKR7A3 or CYP2B6 and expression of p53 protein ($P = 0.006$ and $P < 0.001$, respectively; Table 2) could not be compared with the posttreatment set because of the lack of data on p53 expression in this set.

Patients with higher intratumoral AKR7A3 or CYP2B6 levels than median had significantly longer DFS than those with lower levels (n = 50, DFS: 85.3 vs 68.9 months, $P = 0.032$; and DFS: 93.2 vs 64.1 months, $P = 0.019$, respectively; Figure 2). Multivariate analysis using the Cox regression hazards model with pT, pN, grade, and ER as covariates has confirmed association of high AKR7A3 expression with longer DFS (HR = 3.83, 95% CI = 1.03–14.29, and $P = 0.045$), but not that of CYP2B6 with DFS ($P = 0.083$).

The patients from the pretreatment set were also divided into subgroups according to therapy type. Adjuvant chemotherapy-treated patients (n = 25) with higher intratumoral AKR7A3 or CBR1 levels than median had significantly longer DFS than those with lower levels (DFS: 91.1 vs 57.8 months, $P = 0.040$, and DFS: 89.4 vs 58.1 months, $P = 0.042$, respectively; Figure, Supplemental Digital Content 6, http://links.lww.com/MD/A124). Patients treated with hormone (n = 23) with higher intratumoral CYP3A4 or CBR1 levels than median had significantly shorter DFS than those with lower levels (DFS: 52.8 vs 90.4 months, $P = 0.007$, and DFS: all patients with high CBR1 censored, $P = 0.004$, respectively; Figure, Supplemental Digital Content 6, http://links.lww.com/MD/A124). Because of the low numbers of patients in the compared groups, multivariate analysis was not done and the observed trends have to be interpreted with caution.

Transcript levels of CYP2B6 ($P < 0.001$), CYP3A4 ($P < 0.001$), AKR1C1 ($P < 0.022$) AKR1C2 ($P < 0.001$), and AKR7A3 ($P = 0.001$) were significantly lower in posttreatment tumors compared with the pretreatment ones.

## Protein Expression of AKR1C1, AKR1C2, AKR7A3, CYP2B6, CYP2S1, CYP3A4, and CBR1 in Breast Tumors

Putative markers for which transcript levels significantly associated with response to NACT or DFS of the patients were evaluated at the protein level. Expression of AKR1C1, AKR1C2, AKR7A3, CYP3A4, and CBR1 was assessed by immunoblotting in protein lysates from tumor tissue samples of the independent pretreatment set of patients. No protein of the anticipated size corresponding to CYP2B6 or CYP2S1 was detected in the tumor tissues by the commercially available (CYP2B6) or homemade (CYP2S1) antibodies. CYP2B6 and CYP2S1 protein standards were correctly and quite specifically detected by these antibodies using protein standards and human liver microsomes (Figure, Supplemental Digital Content 7, http://links.lww.com/MD/A124). However, we did not observe protein band comigrating with the standard in all inspected tumors. We regularly detected 2 protein bands with molecular weight by approximately 10–15 kg/mol higher than CYP2B6 there. Bands with different molecular weight than the CYP2S1 standard have also been detected in tumors (Figure, Supplemental Digital Content 8, http://links.lww.com/MD/A124).

The remaining proteins were well detected and quantified by densitometry. GAPDH expression was used as an internal control for normalization of the results. Purified protein standards (CYP2B6, CYP2S1, and CYP3A4), MT-3 cells lysate


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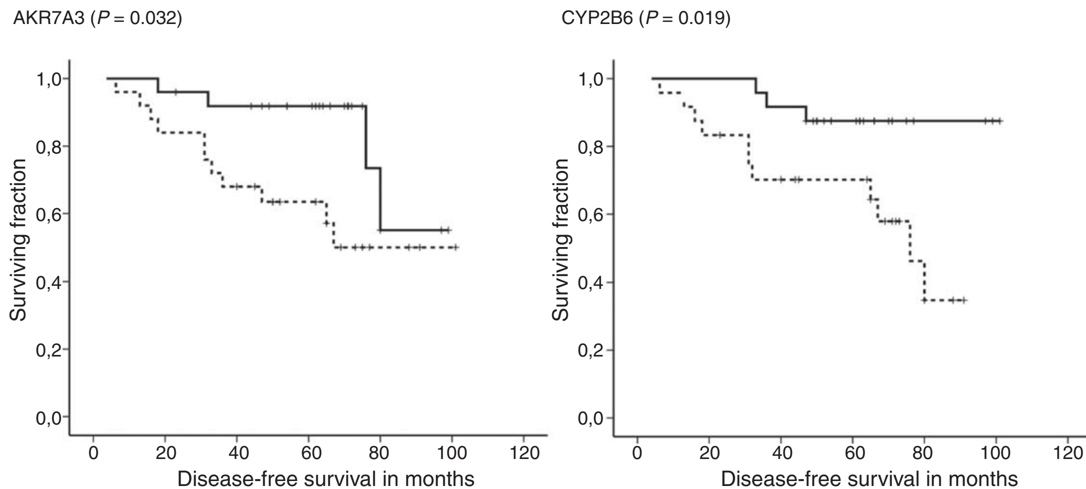


**FIGURE 2.** Associations between gene expression levels and DFS of pretreatment patients. Kaplan–Meier survival curves were plotted for patients ($n = 50$) divided into 2 groups according to the median of transcript levels in tumors. Dashed lines represent the group with lower transcript levels and solid lines represent the group with higher levels than median. Differences between groups were compared using log-rank test. Gene names and significant differences between groups are displayed. All clinical data have been analyzed, but to retain concise style only significant changes are reported. DFS = disease-free survival.

(AKR1C2), human liver lysate (AKR1C1 and CBR1), and a pool of tumor samples (AKR7A3) were used as a calibrator for comparison of variability among membranes. Analysis revealed high interindividual variability in expression of all examined proteins (Figure 3). Protein levels of AKR1C1, AKR7A3, and CBR1 significantly correlated with the respective transcript levels assessed by qPCR in the same tumor samples (Spearman $\rho = 0.47$, $P = 0.003$, Spearman $\rho = 0.61$, $P < 0.001$, and Spearman $\rho = 0.44$, $P = 0.007$, respectively) (Figure 4). The protein levels of CYP3A4 and AKR1C2 did not significantly correlate with the respective transcript levels ($P > 0.05$). Three bands recognized by anti-AKR1C2 antibodies in the anticipated molecular weight range were analyzed by densitometry both separately (not shown) and together (Figure 3) with comparable results, that is, lack of correlation with the transcript level.

## Functional Aspects

AKR1C2 and CYP3A4 were studied in more detail using breast carcinoma model MDA-MB-231 (triple negative) cell line in vitro. In the first experiment, interactions between CYP3A4, AKR1C1, and PCT or adriamycin were addressed. Treatment of the cells with 100 nM PCT resulted in induction of CYP3A4 transcript level, but had no effect on its protein level. AKR1C2 transcript was unaffected, but its protein level was decreased by both 100 nM PCT and 30 $\mu$M adriamycin. Adriamycin had no effect on transcript or protein level of CYP3A4 (Figure 5).

siRNA-directed knockdown of AKR1C2 expression or pcDNA3.1-CYP3A4 plasmid-mediated upregulation of CYP3A4 expression had no effect on proliferation of MDA-MB-231 cells treated by 100 nM PCT (Figure 6). No effect of 30 $\mu$M adriamycin on the MDA-MB-231 proliferation was observed using flow cytometry (results not shown).

## DISCUSSION

Identification of biomarkers with prognostic and predictive value in terms of survival of patients and their response to chemotherapy is a prerequisite step for individualization of cancer treatment.

We studied gene expression of 24 genes coding principal anticancer drug-metabolizing enzymes in tissues from breast carcinoma patients treated by NACT. Our goal was to discover new putative biomarkers with prognostic and predictive value and compare the importance of these biomarkers in 2 groups of patients with different prognoses. In the first phase of analyses, we have identified a number of promising candidates. These candidates were further studied in the set of pretreatment samples and finally protein levels were followed in the third set of samples.

From our observations, we can generalize that the extent of deregulation of gene expression of drug-metabolizing enzymes in tumors of breast carcinoma patients does not strikingly differ between posttreatment and pretreatment patients. However, vast differences between both sets in associations of intratumoral gene expression levels with clinical data of patients observed by this study suggest different prognostic and eventually predictive roles of these particular enzymes.

Taking into consideration the issue of multiple testing, just overexpression of CYP2B6 in tumors expressing hormonal receptors compared with those without such expression is the only universal association typical for both sets. Overexpression of CYP2B6 mRNA in ER-positive breast tumors compared with the normal breast tissue or ER-negative tumors has previously been observed,[25,26] and therefore, our study validates these results on independent sets of patients. We have observed the association of high CYP2B6 mRNA expression with longer DFS of the pretreatment patients for the first time. Although it has previously been shown that ERs regulate CYP2B6 expression in vitro through direct binding to an estrogen responsive element located in the CYP2B6 promoter,[27] we have not detected a protein product of the anticipated molecular weight in breast tumors. Thus, we confirmed the lack of CYP2B6 protein in breast tumors reported by others.[28] In contrast to the published data, we revealed bands of higher molecular weight than the protein standard in all tested samples. The issue of inadequate quality of antibodies is diminished by the fact that

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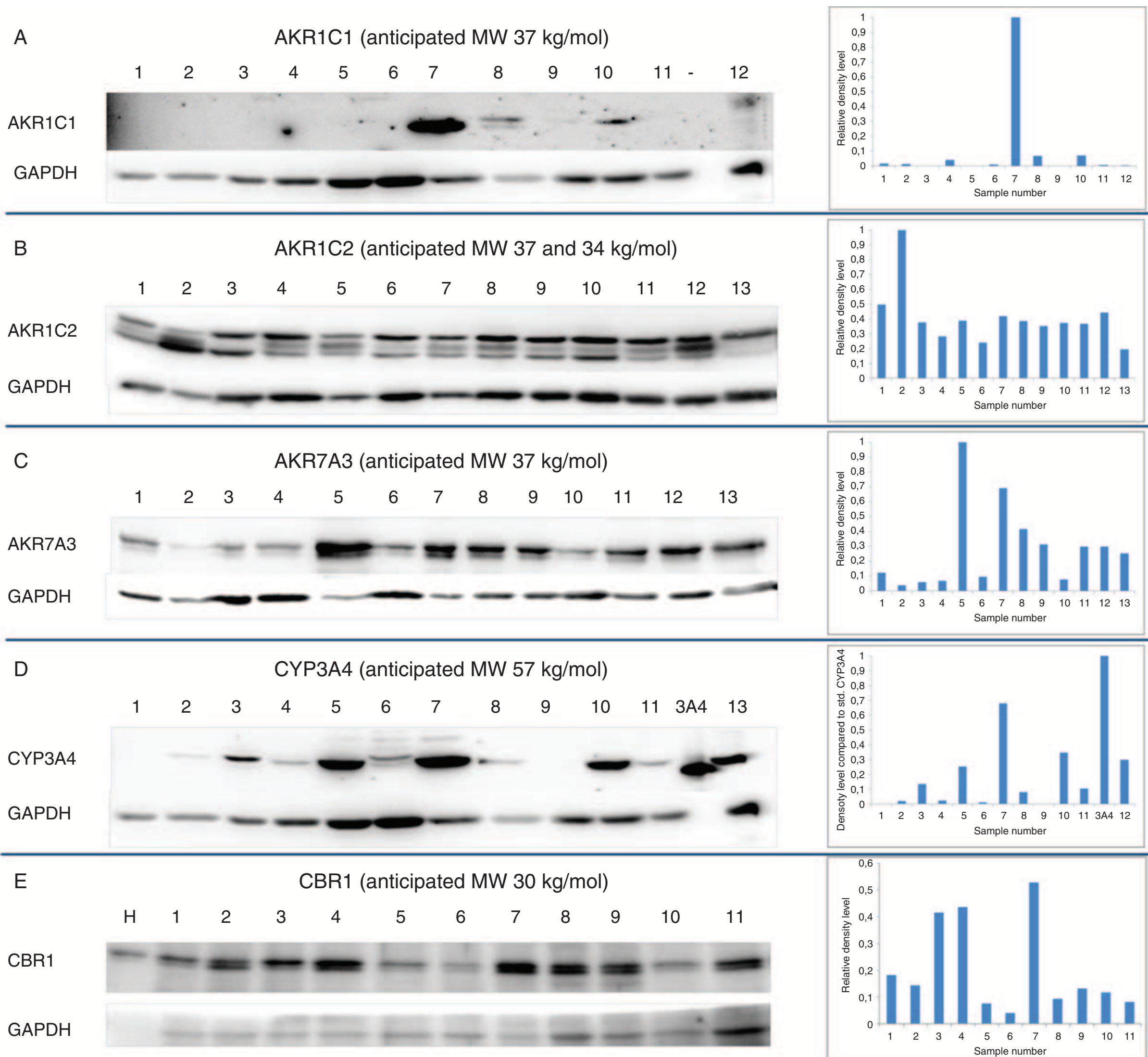


**FIGURE 3.** Protein expression of selected candidates in tumors of breast carcinoma patients. Protein expression of (A) AKR1C1, (B) AKR1C2, (C) AKR7A3, (D) CYP3A4, and (E) CBR1 was assessed by immunoblotting (left part) and evaluated by densitometry with normalization to GAPDH (right part) in representative set of breast tumors as described in the ''Methods.'' Anticipated molecular weight (MW) in kg/mol (in the ±20% range) is presented for each protein according to Human Protein Atlas (http://www.proteinatlas.org). GAPDH = glyceraldehyde phosphate dehydrogenase.

the CYP2B6 protein standard was well recognized and the specificity of antibodies was also good. The nature of the protein bands recognized by anti-CYP2B6 antibodies in breast tumors is being studied.

Associations between expression of hormonal receptors and AKR7A3 were observed although they have not passed correction for multiple testing in both sets. Our data support the higher AKR7A3 protein expression in samples from ER-positive breast carcinoma patients as reported previously.[29] In concordance with the hormonal receptor expression being a factor of more favorable prognosis,[30] high intratumoral AKR7A3 expression was associated with longer DFS of pre-treatment patients in both univariate and multivariate analyses. AKR7A3 protein expression was found in breast tumors for the first time and its high correlation with mRNA levels ($P < 0.001$) demonstrates the biological relevance of AKR7A3 for breast carcinoma. As no other data about the role of AKR7A3 in the prognosis of breast carcinoma patients exist, validation of our findings will be subject of independent follow-up studies.

From other associations found, three may particularly attract further attention. First, responders to NACT had higher intratumoral level of AKR1C2 compared with nonresponders and this association was confirmed by the observed longer DFS in patients with high AKR1C2 level. The association of AKR1C2 with DFS was not observed in the pretreatment patient set suggesting that it may be specific for patients receiving chemotherapy. The present study confirmed the previously observed downregulation of AKR1C2 (and 1C1 and 1C3) in breast tumors compared with nonneoplastic tissues.[31,32] Our study, however, does not comply with the previously published

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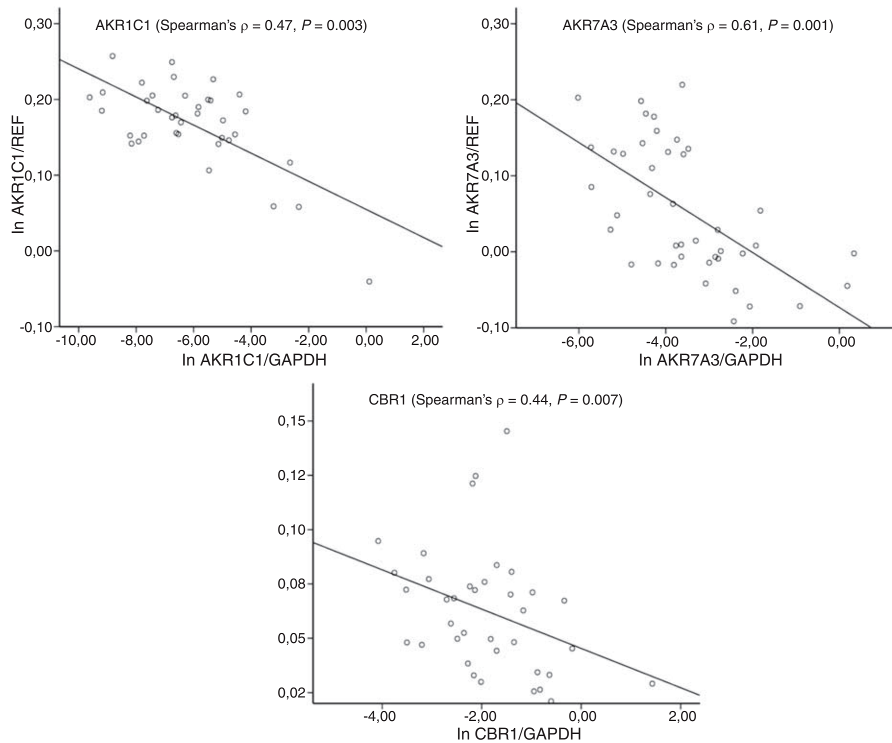


**FIGURE 4.** Correlation between protein and transcript levels. Protein levels were analyzed by densitometry with normalization to GAPDH (*X*-axis) and compared with transcript levels normalized to reference genes (*Y*-axis). Normalized protein and transcript levels were logarithmically normalized before comparison. GAPDH = glyceraldehyde phosphate dehydrogenase.

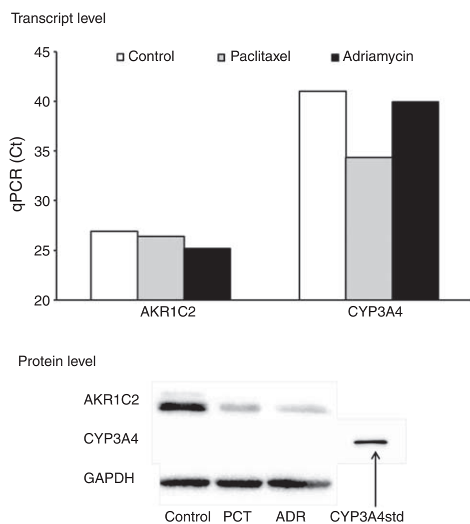


**FIGURE 5.** Interaction between PCT or doxorubicin and protein and transcript levels of CYP3A4 and AKR1C2 in vitro. MDA-MB-231 cell line was incubated without (control) or with 100 nM PCT or 30 μM adriamycin (ADR) as described in the "Methods." qPCR and immunoblotting were done 48 h after the incubation. Ct values from qPCR are presented in the upper part. The higher is the Ct value the lower is the transcript expression. Immunoblots with 10 μg of protein per lane for AKR1C2 and 20 μg of protein or 0.25 pmol of standard for CYP3A4 per lane are presented in the lower part. Two independent experiments were performed with consistent results. Ct = cycle threshold, PCT = paclitaxel, qPCR = quantitative real-time polymerase chain reaction.

data showing that AKR1C2 inhibition by 5β-cholanic acid restored sensitivity of adriamycin-resistant human breast adenocarcinoma cell line breast tumor cells in vitro.[33] We detected AKR1C2 protein in breast carcinomas underlining a potentially functional role of AKR1C2 there. The lack of correlation between AKR1C2 (and CYP3A4) transcript and protein levels observed in the present study may be explained by the use of different normalization controls for qPCR and immunoblotting. The issue of normalization of immunoblotting is a matter of intensive debate.[34] The influence of posttranscriptional processing and protein stability cannot be ignored as well.

In vitro experiments have shown that PCT and adriamycin reduced AKR1C2 protein expression. However, siRNA-directed knockdown of AKR1C2 had no effect on the proliferation of cells treated by PCT. Taken together, the mechanism of action of AKR1C2 in responders to NACT does not seem to be a result of interactions between AKR1C2 and major drugs used in the breast carcinoma treatment regimens.

Second, patients with high intratumoral CYP3A4 level had significantly longer DFS in the posttreatment set. High CYP3A4 transcript[35] or protein[36] levels are predictive for poor response of breast carcinoma patients to docetaxel, which is inactivated by the enzyme. In contrast, CYP3A4 is known as a cyclophosphamide[5]-activating enzyme and from this point of view, the association of high CYP3A4 level with better DFS makes sense in the cyclophosphamide-treated patients (n = 61 in our posttreatment set). In concert with others,[13,28,37] we also found a striking interindividual variability in intratumoral CYP3A4 protein expression among patients. High CYP3A4 protein expression was previously associated with poor survival of breast carcinoma patients.[13] CYP3A4 protein level

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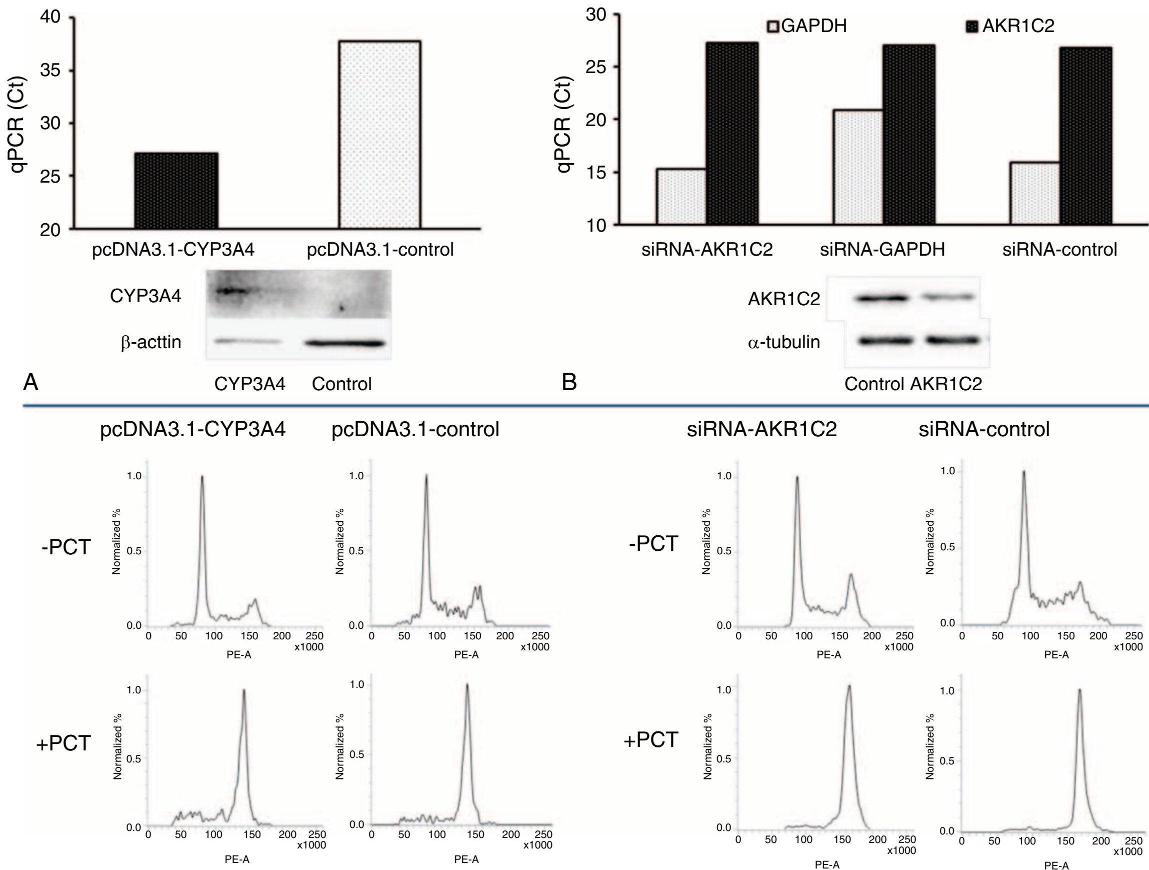


**FIGURE 6.** Influence of AKR1C2 silencing and CYP3A4 induction on MDA-MB-231 cell line proliferation after exposure to PCT in vitro. CYP3A4 expression was induced by transfection MDA-MB-231 cell line with the pcDNA3.1-CYP3A4 plasmid (A) and expression of AKR1C2 was decreased by the siRNA against AKR1C2 (B) as described in the "Methods." Efficiency of cell manipulations was monitored by qPCR and immunoblotting (30 μg of protein per lane for CYP3A4 and 10 μg of protein per lane for AKR1C2). Transcript and protein levels of influenced cells with the respective controls are presented in the upper part. Cells were incubated without (PCT−) or with 100 nM PCT+ for 24 h and then cell proliferation was analyzed using flow cytometry (lower part). Two independent experiments were performed with consistent results. PCT = paclitaxel, siRNA = small interfering RNA.

negatively correlated with the transcript levels in our study, which could explain the observed discrepancy of our results with the published data on the prognostic role of CYP3A4. However, this correlation was insignificant ($P = 0.117$) and therefore the observed association between CYP3A4 and DFS must be cautiously interpreted.

PCT transcriptionally activated CYP3A4, but no induction of P450 3A4 protein was detected in vitro by this study. Thus, the functional relevance of such interaction is quite low if any. Adriamycin had no influence on gene or protein expression of CYP3A4 in vitro. Treatment of cells with enhanced CYP3A4 expression by PCT had no effect on the cell proliferation. However, we were able to induce CYP3A4 transcript level to a high extent, but the protein level was poorly induced in the MDA-MB-231 cell line. CYP3A4 is a subject to ubiquitin-dependent proteasomal degradation by the 26S proteasome, a process involving phosphorylation, ubiquitination, and extraction of endoplasmatic reticulum membrane into the cytosol.[38] Little is known about the nature of these processes in stable cancer cell models as MDA-MB-231. Therefore, for definite answer about the mechanism, in vivo models as mice xenografted with human tumors should be used.

Third, a kind of double-facetted effect was observed for CBR1 in the pretreatment set of patients. A high intratumoral CBR1 level in a chemotherapy-treated subgroup of patients associated with longer DFS, but an opposite effect was found in the hormonal therapy-treated subgroup. These associations have been observed on quite small groups of patients and thus need proper validation in larger cohorts of patients. CBR1 inactivates anthracyclines to the respective alcohols implicated in their cardiotoxicity.[17,39] Besides the fact that *CBR1* genetic polymorphisms have been shown to influence clearance and exposure levels of adriamycin in breast carcinoma patients,[40] just one small study observed no significant difference in CBR1 activity between tumor and normal tissues of breast carcinoma patients.[41] Results of the present study support the recently revealed prognostic significance of decreased CBR1 protein expression (an independent prognostic factor for progression-free and overall survival in multivariate analyses) in endometrial carcinomas.[42] The same authors previously revealed that suppression of CBR1 expression stimulated cancer cell invasion accompanied with the decrease in E-cadherin expression in uterine cervical squamous cell carcinomas.[43] We have no explanation for the

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reversed effect observed in patients treated solely by the hormonal therapy.

We detected CBR1 protein level in all followed tumors and noticed a significant correlation between transcript and protein level. Thus, CBR1 presents another candidate for functional verification in breast carcinoma models.

Additionally, posttreatment patients with high intratumoral CYP2W1 transcript levels responded better to NACT than those with low levels. However, we were unable to validate our results on the nonpreamplified transcript or intratumoral protein levels because of the very low CYP2W1 expression in tumor tissues of the pretreatment set of patients. Thus, CYP2W1 remains an independent biomarker for stages II and III colorectal carcinoma patients,[15] but not for breast carcinoma. Also, the association between AKR1C1 and response to NACT in the posttreatment set could not be verified on the pretreatment set of patients or on the protein level.

## CONCLUSIONS

Associations of AKR1C2, AKR7A3, and CBR1 with prognosis of breast carcinoma patients revealed by this study should be further followed in independent validation and functional studies. The ambiguous roles of CYP2B6 and CYP3A4 noted by this study warrant investigations focused on regulation of their expression and posttranscriptional processing specifically in breast carcinomas.

## ACKNOWLEDGMENTS

*The authors would like to thank Prof Paul F. Hollenberg, University of Michigan, Ann Arbor, MI, and Prof F. Peter Guengerich, Vanderbilt University, Nashville, TN, for their generous gifts of P450 standards for immunoblotting.*

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**Supplemental Digital Content 1:** Primary chemotherapy and hormonal therapy regimens in breast carcinoma patients

| Characteristics | Type | N[a] | % |
|---|---|---|---|
| *Neoadjuvant regimens* (n=68) | Anthracycline in combination[b] | 64 | 95.5 |
| | Hormonal therapy | 3 | 4.5 |
| *Adjuvant regimens* (n=50) | Anthracycline in combination[b] | 23 | 47.9 |
| | CMF only[c] | 2 | 4.2 |
| | Tamoxifen only | 16 | 33.3 |
| | Aromatase inhibitors only | 5 | 10.4 |
| | Tamoxifen & aromatase inhibitors | 2 | 4.2 |

Footnotes:

[a] Information about regimen was not available in one patient from the post-treatment group (neoadjuvant regimens) and in two patients from the pre-treatment group (adjuvant regimens).

[b] Usually FAC/FEC or combination with taxane (AT, ED, etc.).

FAC = 5-fluorouracil/adriamycin/cyclophosphamide

FEC = 5-fluorouracil/epirubicin/cyclophosphamide

[c] CMF=cyclophosphamide/methotrexate/5-fluorouracil combination

**Supplemental Digital Content 2:** Collection and pathological processing of tissue samples

*Patients*

The standard processing of surgical sample and diagnostic histological evaluation was followed according to WHO classification (1). Expression of estrogen and progesterone receptors was assessed according to the published procedure (2) with the 10 % cut-off value. ERBB2 status was defined as positive in samples with immunohistochemical score 2+ or 3+ confirmed by SISH analysis (3).

All patients after primary chemotherapy and surgery were followed for local or distant relapse or in the case of palliative setting for disease progression by regular visits every three months during the first three years, twice a year during the next two years and yearly then after. During the visits mammography, chest X ray, skeletal survey, and abdominal ultrasound was performed yearly and clinical examination together with tumor markers (CEA and CA 15-3) was performed during every visit. In the case of clinical uncertainty additional tests and examinations were performed to rule out possible disease relapse or progression. Response to NACT was evaluated by RECIST criteria as described (4).

*Immunohistochemical detection of p53 protein expression*

Fresh tissue samples of the mammary tumors were fixed in standard neutral buffered 4 % formaldehyde for up to 26 hours and embedded into paraffin with classical histological techniques. For immunohistochemical investigation 3 µm thick histological sections were utilized. Primary antibody against the p53 (clone DO-7; monoclonal mouse antibody detecting both mutant and wild type p53 protein) was purchased from Dako (Dako, Glostrup, Denmark). Antibodies were diluted with Dako Antibody diluent (1:50). For p53 detection, the sections were further processed with heat-induced epitope retrieval in 10 mmol/l citrate buffer pH 6.0 in water bath (40 min heating at 95-99° C and then 20 min cooling at room temperature). Tissues were incubated with primary antibodies overnight at 4° C. Detection was performed with peroxidase/diaminobenzidine system. Evaluation of binding of both primary antibodies was performed with Dako REAL Detection System (LSAB+, biotinylated secondary goat anti-mouse antibodies/streptavidin conjugated to horseradish peroxidase). As a chromogen, 0.04 % DAB (3,3´-diaminobenzidine tetrahydrochloride dihydrate; Fluka, Buchs, Switzerland) in 50 mmol/l TRIS (Tris-hydroxymethyl amino methane)/0.015 % $H_2O_2$ was used. Several p53 positive cells were present in each sample analyzed. p53 status was

evaluated as positive, if more than 50 % of tumor cells were immunohistochemically stained according to the previously published evaluation procedure (5, 6).

*Selection of reference genes*

For selection of reference genes, 96-well TaqMan Array Plates (Life Technologies) were used and evaluated as previously published (7, 8). EIF2B1, MRPL19, UBB, and IPO8 were selected as the most stable reference genes for data normalization using geNorm and NormFinder programs (9, 10).

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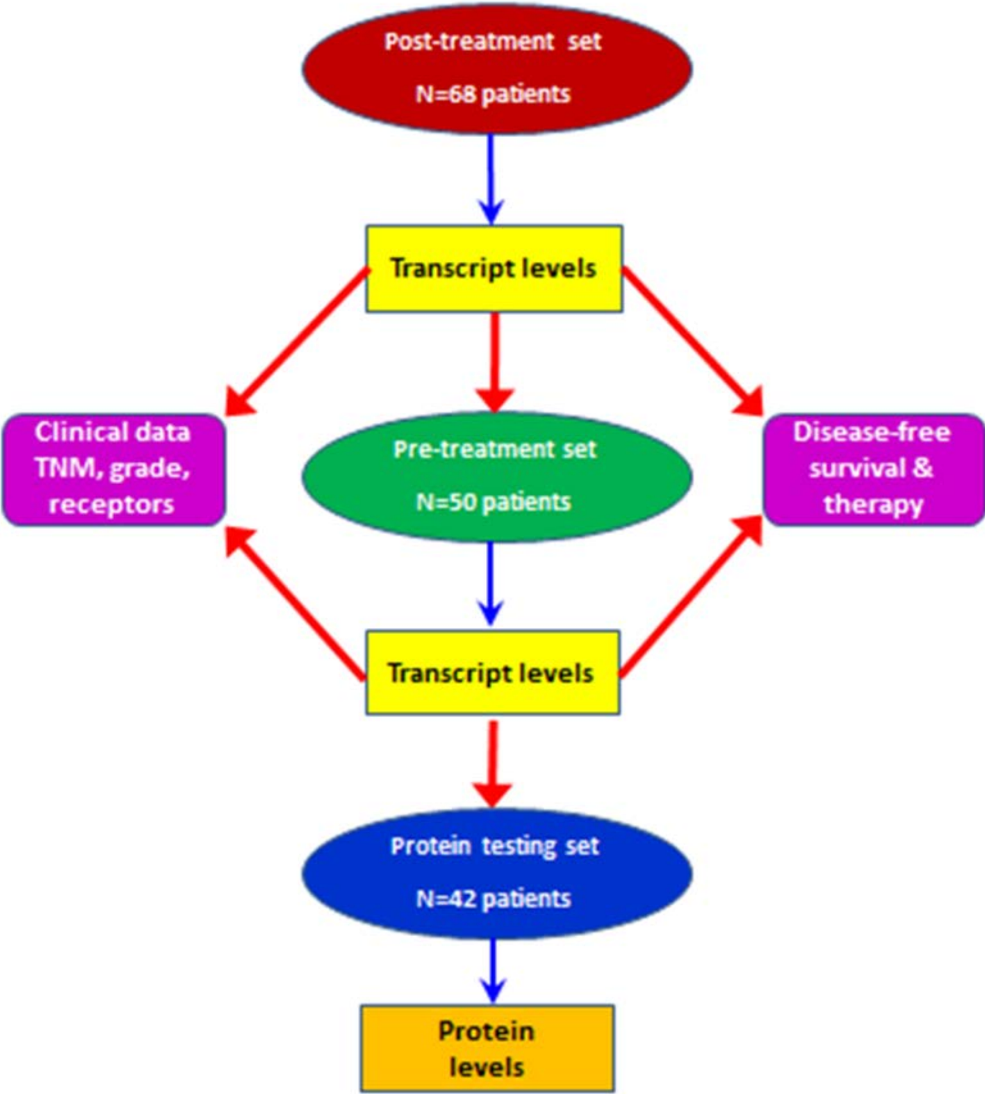
**Supplemental Digital Content 3:** Flow diagram of the study




Experimental procedures are displayed by blue lines and selection process and statistical analyses by red lines.

**Supplemental Digital Content 4:** Clinical characteristics of breast carcinoma patients

| Characteristics | Post-treatment set[a] | | Pre-treatment set[a] | |
|---|---|---|---|---|
| *Age at diagnosis*, mean ± SD (years) | 53.0 ± 11.5 | | 61.5 ± 10.2 | |
| *Menopausal status* | | | | |
| Premenopausal | 31 | (46) | 7 | (14) |
| Postmenopausal | 37 | (54) | 43 | (86) |
| Tumor size, mean ± SD (mm) | 21.0 ± 14.7 | | 18.9 ± 12.3 | |
| *Lymph node metastasis* | | | | |
| Absent (pN0) | 41 | (60) | 23 | (48) |
| Present (pN1-3) | 27 | (40) | 25 | (52) |
| pNx | 0 | - | 2 | - |
| *Pathological stage* | | | | |
| SI | 24 | (37) | 17 | (35) |
| SII | 34 | (52) | 21 | (44) |
| SIII | 7 | (11) | 10 | (21) |
| Not determined | 3 | - | 2 | - |
| *Histological type* | | | | |
| Invasive ductal carcinoma | 57 | (84) | 41 | (82) |
| Other type | 11 | (16) | 9 | (18) |
| *Pathological grade* | | | | |
| G1 | 8 | (12) | 11 | (13) |
| G2 | 29 | (44) | 28 | (58) |
| G3 | 29 | (44) | 9 | (19) |
| Gx | 2 | - | 3 | - |
| *Estrogen receptor status* | | | | |
| Positive | 47 | (69) | 37 | (74) |
| Negative | 21 | (31) | 13 | (26) |
| *Progesterone receptor status* | | | | |
| Positive | 48 | (71) | 32 | (64) |
| Negative | 20 | (29) | 18 | (36) |
| *Expression of ERBB2* | | | | |
| Positive | 16 | (24) | 11 | (22) |
| Negative | 51 | (76) | 39 | (78) |
| Unknown | 1 | - | 0 | - |
| *Expression of Ki-67*, mean ± SD (%) | 32.6 ± 23.1 | | not available | |
| Unknown | 1 | - | not available | |
| *Expression of p53* | | | | |
| Positive | not available | | 12 | (25) |
| Negative | not available | | 37 | (76) |
| Unknown | not available | | 4 | - |
| *Response* | | | | |
| Partial response | 38 | (60) | not applicable | |
| Stable disease or progression | 25 | (40) | not applicable | |
| Not assessed | 5 | - | not applicable | |

Footnotes:

[a] Number of patients with % in parentheses; SD = Standard deviation

**Supplemental Digital Content 5:** List of TaqMan Gene Expression Assays used in the study

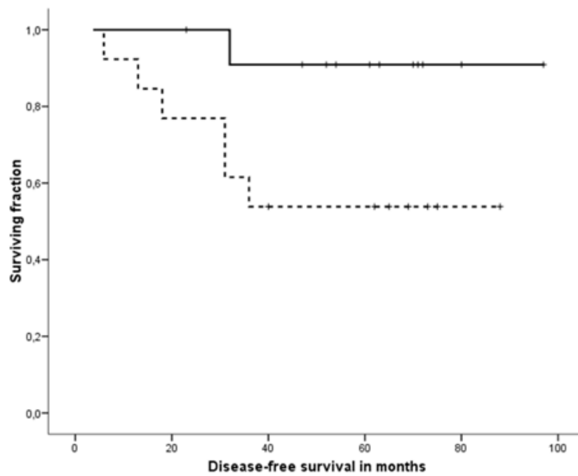
| Gene symbol | Assay ID | Accession number | Exon boundary | Amplicon length | PCR efficiency |
|---|---|---|---|---|---|
| **REFERENCE GENES** | | | | | |
| EIF2B1 | Hs00426752_m1 | NM_001414.3 | 4 – 5 | 75 | 0.94 |
| IPO8 | Hs00183533_m1 | NM_006390.3 | 20 – 21 | 71 | 0.94 |
| MRPL19 | Hs00608519_m1 | NM_014763.3 | 2 – 3 | 72 | 0.93 |
| UBB | Hs00430290_m1 | NM_018955.2 | 1 – 2 | 120 | 0.96 |
| **CYTOCHROMES P450** | | | | | |
| CYP1A2 | Hs00167927_m1 | NM_000761.3 | 2 – 3 | 67 | 1.24 |
| CYP2B6 | Hs03044634_m1 | NM_000767.4 | 6 – 7 | 120 | 1.02 |
| CYP2C8 | Hs00258314_m1 | M17398.1 | 7 – 8 | 108 | 0.92 |
| CYP2C9 | Hs02383631_s1 | NM_000771.3 | 9 – 9 | 91 | 1.03 |
| CYP2C19 | Hs00426380_m1 | NM_000769.1 | 5 – 6 | 106 | 0.82 |
| CYP2D6 | Hs00164385_m1 | NM_000106.5 | 2 – 3 | 74 | 1.02 |
| CYP2S1 | Hs00258076_m1 | NM_030622.6 | 2 – 3 | 55 | 0.92 |
| CYP3A4 | Hs00430021_m1 | NM_017460.5 | 8 – 9 | 92 | 0.96 |
| CYP3A5 | Hs00241417_m1 | NM_000777.3 | 3 – 4 | 82 | 0.97 |
| CYP2W1 | Hs00214994_m1 | NM_017781.2 | 2 – 3 | 55 | 0.87 |
| **ALDO–KETO REDUCTASES** | | | | | |
| AKR1A1 | Hs00195992_m1 | NM_153326.2 | 2 – 3 | 103 | 0.97 |
| AKR1B1 | Hs00739326_m1 | NM_001628.2 | 4 – 5 | 139 | 0.97 |
| AKR1B10 | Hs00252524_m1 | NM_020299.4 | 3 – 4 | 95 | 0.96 |
| AKR1C1 | Hs00413886_m1 | NM_001353.5 | 8 – 9 | 103 | 0.97 |
| AKR1C2 | Hs00912742_m1 | NM_205845.2 | 1 – 2 | 92 | 1.08 |
| AKR1C3 | Hs00366267_m1 | NM_003739.4 | 8 – 9 | 112 | 0.93 |
| AKR1C4 | Hs00559542_m1 | NM_001818.3 | 7 – 8 | 123 | NA |
| AKR1D1 | Hs00818881_m1 | NM_005989.3 | 1 – 2 | 103 | NA |
| AKR7A2 | Hs00761005_s1 | NM_003689.3 | 7 – 7 | 114 | 0.97 |
| AKR7A3 | Hs00792041_gH | NM_012067.2 | 5 – 6 | 65 | 1.00 |
| KCNAB1 | Hs00185764_m1 | NM_172159.3 | 6 – 7 | 79 | 0.99 |
| KCNAB2 | Hs00186308_m1 | NM_172130.2 | 4 – 5 | 68 | 0.98 |
| KCNAB3 | Hs00190986_m1 | NM_004732.2 | 7 – 8 | 54 | 0.98 |
| **CARBONYL REDUCTASE 1** | | | | | |
| CBR1 | Hs00156323_m1 | NM_001757.2 | 2 – 3 | 73 | 0.96 |

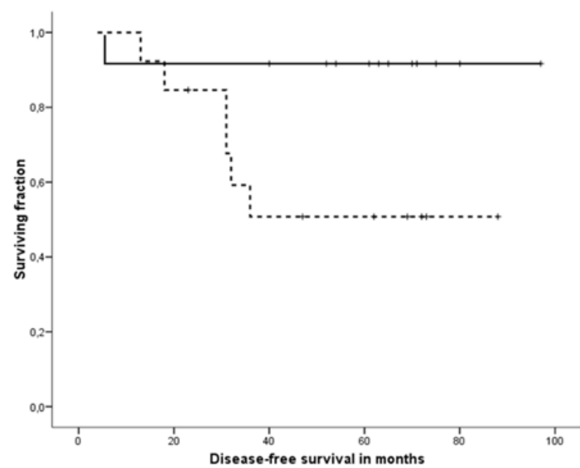
Footnote:
NA = not applicable - PCR amplification efficiency could not be estimated due to the low expression level

**Supplemental Digital Content 6:** Associations between gene expression levels and DFS of pre-treatment patients divided by therapy type

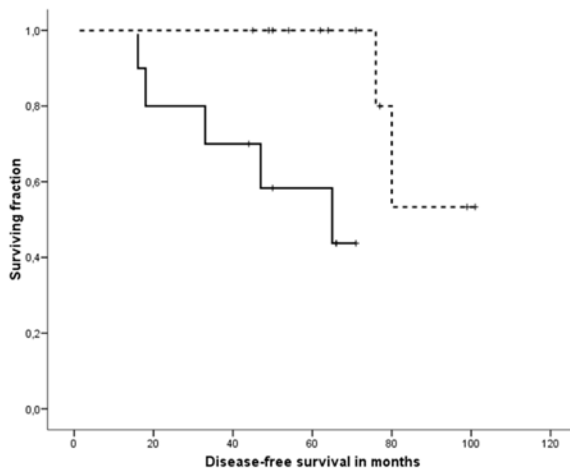
Kaplan-Meier survival curves were plotted for patients treated by chemotherapy (**A**, n = 25) or by hormonal therapy (**B**, n = 23) divided into two groups according to the median of transcript levels in tumors. Dashed lines represent the group with lower transcript levels and solid lines represent the group with higher levels than median. Differences between groups were compared using Log-rank test. The gene name and significant difference between groups are displayed.

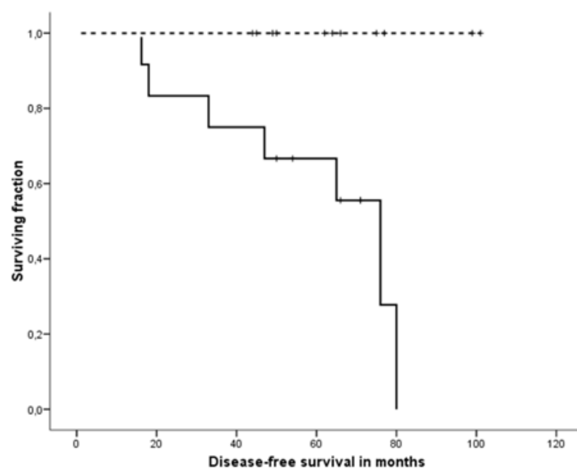
**A** AKR7A3 ($P = 0.040$) CBR1 ($P = 0.042$)




**B** CYP3A4 ($P = 0.007$) CBR1 ($P = 0.004$)



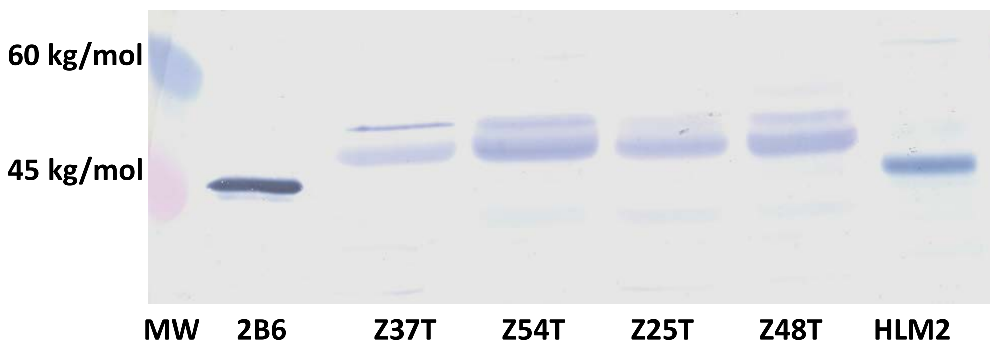
**Supplemental Digital Content 7:** Immunoblot of CYP2B6 in human breast carcinomas (described in Methods).

**A – Antibody specificity test**

MW = molecular weight marker, HLM2 = human liver microsomes (10 µg/lane), 1A2, 2A6, 2C9, 2D6, 2E1, 3A4, and 2B6 (purified P450 protein standards, 1 pmol/lane).

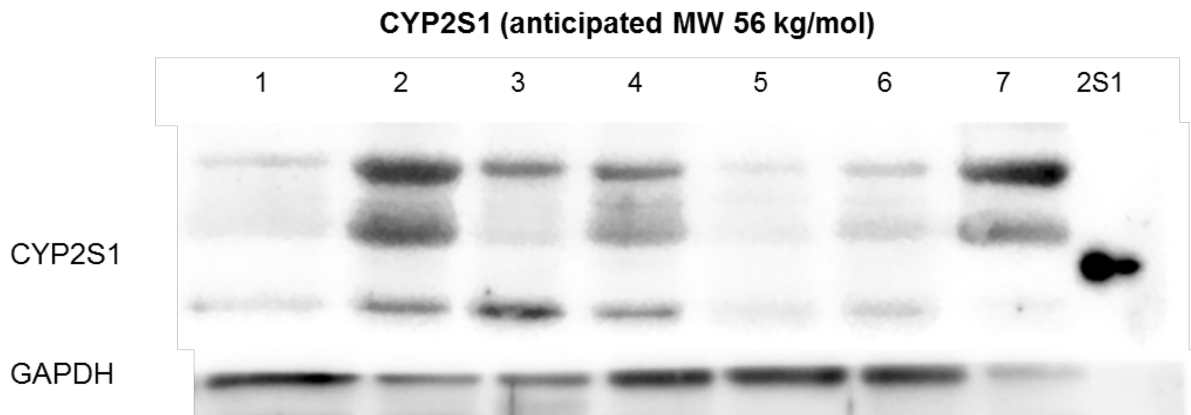
**B – Tissue test**

MW = molecular weight marker, HLM2 = human liver microsomes (10 µg/lane), 2B6 (purified P450 protein standard, 1 pmol/lane), Z37T, Z54T, Z25T, Z48T = tumor tissues from four patients (30 µg/lane).




Resume: Antibodies recognized strongly CYP2B6 and weakly CYP2A6 proteins (A). Protein corresponding to the correct molecular weight was recognized by anti-CYP2B6 antibodies in human liver microsomes but not in tumor tissues (B). Protein bands of higher molecular weight were observed in tumors.

**Supplemental Digital Content 8:** Immunoblot of CYP2S1 in human breast carcinomas (described in Methods).




2S1 = purified P450 protein standard (0.25 pmol/lane), 1 - 7 = tumor tissues from seven patients (30 µg/lane).

Resume: Protein corresponding to the correct molecular weight was recognized by anti-CYP2S1 antibodies only in standard but not in tumor tissues. Protein bands of higher and lower molecular weight were observed in tumors.

## Příloha č. 4:

Kunická T, Václavíková R, **Hlaváč V**, Vrána D, Pecha V, Rauš K, Trnková M, Kubáčková K, Ambruš M, Vodičková L, Vodička P, Souček P.

**Non-Coding Polymorphisms in Nucleotide Binding Domain 1 in ABCC1 Gene Associate with Transcript Level and Survival of Patients with Breast Cancer**

PLoS ONE 2014;9(7): e101740 [IF: 3,53] 9 % *)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

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# Non-Coding Polymorphisms in Nucleotide Binding Domain 1 in *ABCC1* Gene Associate with Transcript Level and Survival of Patients with Breast Cancer

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## Abstract

***Objectives:*** ATP-Binding Cassette (ABC) transporters may cause treatment failure by transporting of anticancer drugs outside of the tumor cells. Multidrug resistance-associated protein 1 coded by the *ABCC1* gene has recently been suggested as a potential prognostic marker in breast cancer patients. This study aimed to explore tagged haplotype covering nucleotide binding domain 1 of *ABCC1* in relation with corresponding transcript levels in tissues and clinical phenotype of breast cancer patients.

***Methods:*** The distribution of twelve *ABCC1* polymorphisms was assessed by direct sequencing in peripheral blood DNA (n = 540).

***Results:*** Tumors from carriers of the wild type genotype in rs35623 or rs35628 exhibited significantly lower levels of ABCC1 transcript than those from carriers of the minor allele (p = 0.003 and p = 0.004, respectively). The ABCC1 transcript levels significantly increased in the order CT-GT>CC-GT>CC-GG for the predicted rs35626-rs4148351 diplotype. Chemotherapy-treated patients carrying the T allele in rs4148353 had longer disease-free survival than those with the GG genotype (p = 0.043). On the other hand, hormonal therapy-treated patients with the AA genotype in rs35628 had significantly longer disease-free survival than carriers of the G allele (p = 0.012).

***Conclusions:*** Taken together, our study shows that genetic variability in the nucleotide binding domain 1 has a significant impact on the ABCC1 transcript level in the target tissue and may modify survival of breast cancer patients.

**Citation:** Kunická T, Václavíková R, Hlaváč V, Vrána D, Pecha V, et al. (2014) Non-Coding Polymorphisms in Nucleotide Binding Domain 1 in *ABCC1* Gene Associate with Transcript Level and Survival of Patients with Breast Cancer. PLoS ONE 9(7): e101740. doi:10.1371/journal.pone.0101740

**Editor:** Robert Lafrenie, Sudbury Regional Hospital, Canada

**Received** February 12, 2014; **Accepted** June 10, 2014; **Published** July 31, 2014

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**Funding:** This work was supported by grant of the Internal Grant Agency of the Czech Ministry of Health (www.mzcr.cz), no.: NT13679-4 and by the project of the Czech Science Foundation no.: P303/12/G163 (www.gacr.cz). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Biolab Praha, k.s provided support in the form of salary for author MT, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific role of this author is articulated in the 'author contributions' section.

**Competing Interests:** Dr. Marketa Trnková is employed by a commercial company (Biolab Praha, k.s.). This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

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## Introduction

Breast cancer (OMIM: 114480) is the most common malignancy affecting female population worldwide. Despite early detection and improved understanding of molecular mechanisms of this disease, it is still the second leading cause of cancer death in women [1].

Multidrug resistance (MDR) represents a major obstacle to successful therapy of tumors. MDR was first described in 1970 [2] as a cross-resistance to structurally and functionally different anticancer drugs. Most MDR is caused by enhanced expression of membrane-bound ATP-Binding Cassette (ABC) transporters [3,4]. ABC transporters pump drugs outside of the cells into the extracellular space, thus reducing their cytotoxic effect [5–7].

Multidrug resistance-associated protein 1 (MRP1/ABCC1, OMIM: 158343) was the first identified member of the ABCC subfamily [8]. *ABCC1* gene is located on the 16$^{th}$ chromosome at position p13.11, is approximately 200 kb long, comprises 31 exons, and encodes 190 kDa membrane protein comprising 1531 amino acids [9]. ABCC1 transports a number of physiological substrates (glutathione, leucotrienes, prostaglandins, etc.) and xenobiotics including anticancer drugs (anthracyclines, taxanes, methotrexate,

*Vinca* alkaloids, camptothecins, etc.) [10]. The involvement of ABCC1 in the resistance to chemotherapy has been reported in various types of solid tumors [11].

A recent tissue microarray study has concluded that high ABCC1 protein expression is a negative prognostic marker as it has been found in highly aggressive molecular subtypes of breast carcinoma [12]. Significant overexpression of ABCC1 transcript in both pre-chemotherapy (n = 100) and post-chemotherapy (n = 68) tumors compared with adjacent non-neoplastic tissues from breast carcinoma patients and associations of intratumoral transcript levels with tumor grade and expression of estrogen receptor, proliferative marker Ki67, and p53 protein have been recently reported [13].

A high number of single nucleotide polymorphisms (SNPs) in *ABCC1* have been identified in different human populations and their haplotypes were examined [14,15]. *ABCC1* has high haplotype diversity with significant differences across ethnic groups [16]. Very recently convincing association between rs4148350, rs45511401, and rs246221 SNPs in *ABCC1* and risk of febrile neutropenia in breast cancer patients treated by 5-fluorouracil, epirubicin and cyclophosphamide (FEC regimen) has been shown [17]. Several studies suggested *in vitro* functional effects of SNPs in *ABCC1*. For instance, Gly671Val (dbSNP: rs45511401) SNP located near the nucleotide binding domain 1 (NBD1, **Figure 1**) which is important for the ATPase activity was associated with reduced levels of ABCC1 transcript [14]. Serine at position 433 (rs60782127) significantly increased the resistance to doxorubicin [18] whereas serine at position 43 (rs41395947) enhanced expression and altered ABCC1 protein trafficking to the plasma membrane [19]. Moreover, several *ABCC1* SNPs including Arg723Gln (rs4148356) located between the Walker A and B motifs in NBD1 have been shown to affect the resistance to a number of anticancer drugs [20].

The present study investigated the effect of tagged haplotype of the *ABCC1* gene covering NBD1 with adjacent sequences at ABCC1 transcript level in tumor and non-neoplastic tissues from breast cancer patients. In addition, we also addressed the prognostic and predictive significance of genetic variability of *ABCC1*.

## Materials and Methods

### Material

General chemicals, histopaque (Ficoll), phenol, chloroform, RNase A, proteinase K, *Taq* polymerase, and histidine were purchased from Sigma-Aldrich (Prague, Czech Republic). Deoxynucleotides (dNTPs) for PCR and molecular weight standards for electrophoresis (ΦX174DNA/HaeIII digest) were products of New England Biolabs, Inc. (Ipswich, MA). Ultrapure agarose was supplied by Life Technologies (Prague, Czech Republic).

### Patients

The study included a total of 540 breast cancer patients (C50 according to ICD-10) of Caucasian origin diagnosed in Motol Faculty Hospital, Institute for the Care for Mother and Child, BIOLAB Praha k.s., and Faculty Hospital Kralovske Vinohrady in Prague, Czech Republic between February 2000 and December 2010 (for study flow diagram see **Figure S1 in File S1**). Blood samples were available from all patients. Tumor tissue samples were collected during the primary surgery from subgroups of patients. First subgroup of patients (n = 60) underwent preoperative neoadjuvant chemotherapy regimens based on 5-fluorouracil/anthracyclines/cyclophosphamide (FAC or FEC) and/or taxanes. The second subgroup was treated by adjuvant chemotherapy and/or hormonal therapy after surgery (n = 89). Paired samples of adjacent non-neoplastic tissues as controls were available from 67 patients. In the whole set, patients with metastatic disease treated by first line palliative therapy were also included (for all treatments see **Table S1 in File S1**). Collection and pathological processing of tissue samples and retrieval of data was performed as described before [13,21]. Expression of receptors for estrogen (ER) and progesterone (PR) was evaluated as positive when at least 10% of cell nuclei showed staining by routine immunohistochemistry. HER2 (ERBB2, OMIM: 164870) status was defined as positive in samples with immunohistochemical score 2+ or 3+ confirmed by SISH analysis. For expression of the p53 (OMIM: 191170) protein, 50% cut off was used (negative <50% vs. positive ≥50%, see Material and Methods S1 and References S1 in File S1). Patients were experimentally divided into groups according to molecular subtypes of their tumors (Luminal A = ER+/HER2− and grade 1 or 2, Luminal B/HER2- = ER+/HER2− and grade

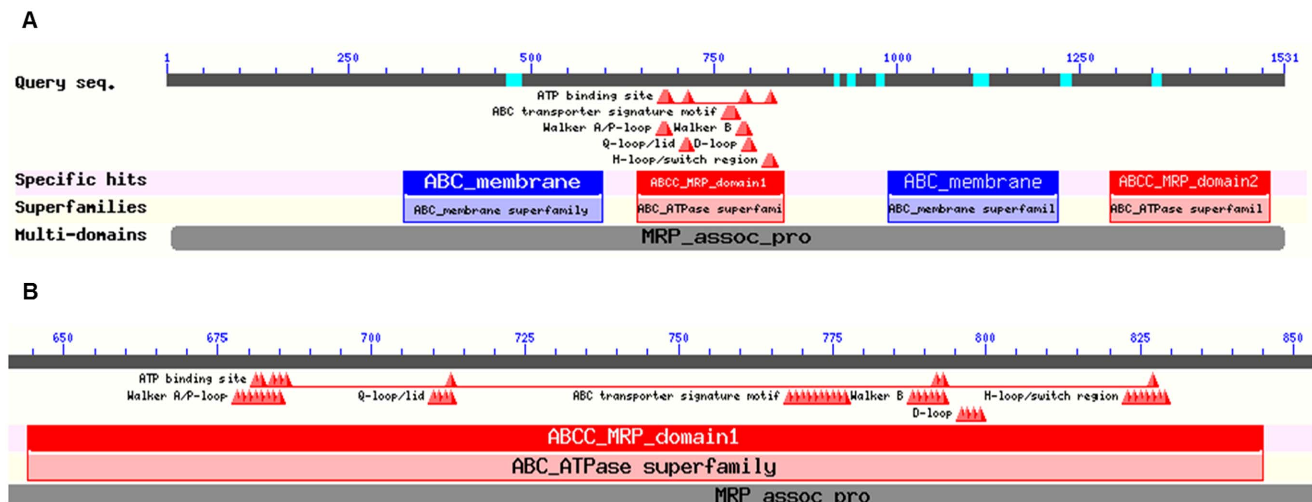


**Figure 1. Schematic representation of functional domains of ABCC1.** Figure depicts functional domains of ABCC1 protein (A) and important structural motifs within NBD1 (B). Data modified from NCBI's Conserved Domain Database (CDD) [49].
doi:10.1371/journal.pone.0101740.g001

**Table 1.** Clinical-pathological characteristics of patients.

| Characteristics | Type | n | % |
|---|---|---|---|
| **Average age at diagnosis** | 58±11 years | 540 | 100.0 |
| **Menopausal status** | premenopausal | 119 | 22.2 |
| | postmenopausal | 416 | 77.8 |
| | not assessed | 5 | – |
| **Histological tumor type** | invasive ductal | 400 | 76.0 |
| | other invasive type | 126 | 24.0 |
| | not assessed | 14 | – |
| **Histological grade (G)** | GI | 103 | 22.1 |
| | GII | 238 | 51.1 |
| | GIII | 125 | 26.8 |
| | Gx | 74 | – |
| **Stage (S)** | SI | 223 | 44.7 |
| | SII | 211 | 42.3 |
| | SIII | 51 | 10.2 |
| | SIV | 14 | 2.8 |
| | not assessed | 41 | – |
| **pT** | pT1 | 316 | 61.5 |
| | pT2 | 161 | 31.3 |
| | pT3 | 17 | 3.3 |
| | pT4 | 20 | 3.9 |
| | pTx | 26 | – |
| **pN** | pN0 | 316 | 62.0 |
| | pN1 | 158 | 31.0 |
| | pN2 | 25 | 4.9 |
| | pN3 | 11 | 2.2 |
| | pNx | 30 | – |
| **cM** | cM0 | 501 | 97.1 |
| | cM1 | 15 | 2.9 |
| | cMx | 24 | – |
| **Expression of estrogen** | positive | 393 | 74.9 |
| **receptor** | negative | 132 | 25.1 |
| | not assessed | 15 | – |
| **Expression of** | positive | 385 | 73.8 |
| **progesterone receptor** | negative | 137 | 26.2 |
| | not assessed | 18 | – |
| **Expression/amplification** | positive | 120 | 25.2 |
| **of HER2** | negative | 357 | 74.8 |
| | not assessed | 63 | – |
| **p53 protein expression** | positive | 35 | 29.9 |
| | negative | 82 | 70.1 |
| | not assessed | 423 | – |

doi:10.1371/journal.pone.0101740.t001

3, Luminal B/HER2+ = ER+/HER2+, HER2+ = ER−/HER2+, and triple negative = ER−/PR−/HER2−) according to [22]. All patients after primary chemotherapy and surgery were followed for local or distant relapse or in the case of palliative setting for disease progression by regular visits every 3 months during the first 3 years, twice a year during the next 2 years and yearly then after. During the visits mammography, chest X ray, skeletal survey, and abdominal ultrasound was performed yearly and clinical examination together with tumor markers (CEA and CA 15-3) was performed during every visit. In the case of clinical uncertainty, additional tests and examinations were performed to rule out possible disease relapse or progression.

## Ethics statement

All patients were asked to read and sign an informed consent and the study was approved by the Ethical Commission of the National Institute of Public Health in Prague.

## DNA extraction

Blood samples were collected during the diagnostic procedures using tubes with $K_3EDTA$ anticoagulant. Genomic DNA was isolated from human peripheral blood lymphocytes by the standard phenol/chloroform extraction and ethanol precipitation method [23]. DNA samples were stored in aliquots at −20°C prior to analysis.

## ABCC1 genotyping

DNA sequence covering coding exons 15–19 (NBD1), interspersed introns, and sequences surrounding both 5′- and 3′-untranslated regions (Chr16:16,076,000–16,091,000, NCBI Build 36.3 version) were analyzed by HaploView v4.2 program [24]. Together nine SNPs tagging common haplotypes at $r^2$>0.8 and

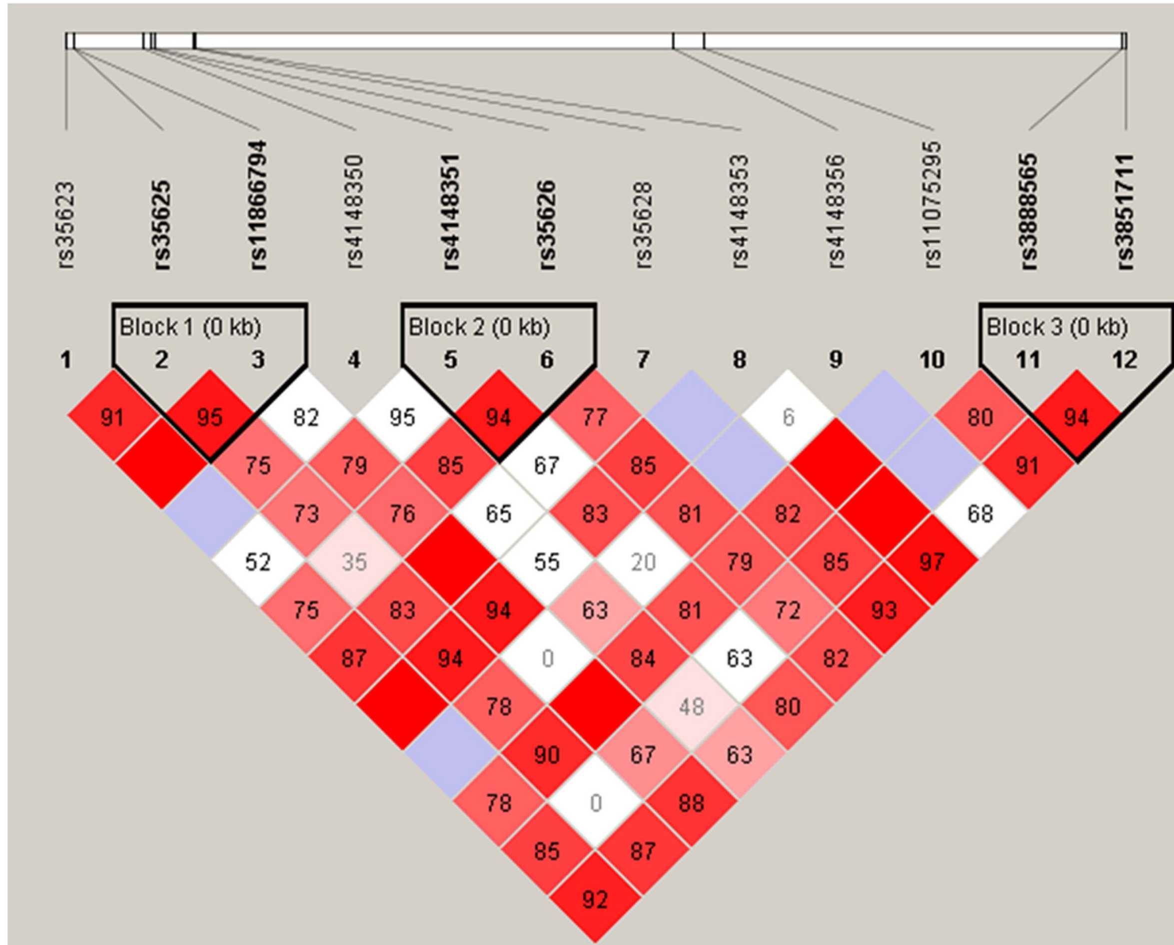

**Figure 2. Haplotype analysis of *ABCC1* SNPs.** Figure indicates linkage disequilibrium plot (A) and three blocks comprising of SNP diplotypes (B) predicted from experimental data obtained in the present study. The likelihood of linkage of two tested SNPs increases from white to red color (A). Population frequency of diplotypes and connections from one diplotype block to the next one are shown (B). Analysis was performed by HaploView v4.2 program.

doi:10.1371/journal.pone.0101740.g002

**Table 2.** Distribution of *ABCC1* SNPs and allele frequencies in breast cancer patients.

| SNP ID | Localization | Genotype | distribution | | Missing genotypes | Type* | MAF# |
|---|---|---|---|---|---|---|---|
| | | Genotype | n | % | n (%) | | |
| **rs35623** | intron 15 | GG | 422 | 79.5 | 9 (1.7) | NC | T (0.11) |
| | | GT | 100 | 18.8 | | | |
| | | TT | 9 | 1.7 | | | |
| **rs35625** | intron 15 | TT | 217 | 40.9 | 9 (1.7) | NC | C (0.37) |
| | | TC | 234 | 44.1 | | | |
| | | CC | 80 | 15.1 | | | |
| **rs11866794** | intron 15 | GG | 415 | 78.2 | 9 (1.7) | NC | C (0.12) |
| | | GC | 108 | 20.3 | | | |
| | | CC | 8 | 1.5 | | | |
| **rs4148350** | intron 15 | GG | 466 | 89.3 | 18 (3.3) | NC | T (0.06) |
| | | GT | 52 | 10.0 | | | |
| | | TT | 4 | 0.8 | | | |
| **rs4148351** | intron 15 | CC | 416 | 79.5 | 17 (3.1) | NC | T (0.11) |
| | | CT | 97 | 18.6 | | | |
| | | TT | 10 | 1.9 | | | |
| **rs35626** | intron 16 | GG | 261 | 50.1 | 19 (3.5) | NC | T (0.29) |
| | | GT | 220 | 42.2 | | | |
| | | TT | 40 | 7.7 | | | |
| **rs35628** | intron 16 | AA | 441 | 84.0 | 15 (2.8) | NC | G (0.09) |
| | | AG | 77 | 14.7 | | | |
| | | GG | 7 | 1.3 | | | |
| **rs4148353** | intron 16 | GG | 426 | 81.3 | 16 (3.0) | NC | T (0.10) |
| | | GT | 91 | 17.4 | | | |
| | | TT | 7 | 1.3 | | | |
| **rs4148356** | exon 17 | GG | 516 | 96.3 | 4 (0.7) | R723Q | A (0.02) |
| | | GA | 19 | 3.5 | | | |
| | | AA | 1 | 0.2 | | | |
| **rs11075295** | intron 17 | AA | 375 | 69.8 | 3 (0.6) | NC | G (0.17) |
| | | AG | 139 | 25.9 | | | |
| | | GG | 23 | 4.3 | | | |
| **rs3888565** | intron 18 | GG | 380 | 70.6 | 2 (0.4) | NC | A (0.17) |
| | | GA | 134 | 24.9 | | | |
| | | AA | 24 | 4.5 | | | |

**Table 2.** Cont.

| SNP ID | Localization | Genotype | Genotype distribution | | Missing genotypes | Type* | MAF# |
|---|---|---|---|---|---|---|---|
| | | Genotype | n | % | n (%) | | |
| **rs3851711** | intron 18 | TT | 151 | 28.1 | 2 (0.4) | NC | G (0.49) |
| | | TC | 248 | 46.1 | | | |
| | | CC | 139 | 25.8 | | | |

Footnotes:
*NC = non-coding.
#MAF = minor allele frequency.
doi:10.1371/journal.pone.0101740.t002

minor allele frequency (MAF)>0.05 in HapMap CEU sample with minimally 75% genotype data were identified. The *ABCC1* region containing eight selected SNPs (rs35623, rs4148351, rs35626, rs11075295, rs3851711, rs3888565, rs35625, and rs4148350) was then divided into four regions. Inside of these regions, we also analyzed additional four SNPs (rs35628, rs11866794, rs4148353, and rs4148356). All analyzed SNPs are characterized in **Table S2 in File S1.** For each region pair of forward and reverse primers with M13 sequence adaptors was designed using the Primer3 software [25]. Oligonucleotide primers were synthesized by Generi Biotech (Hradec Kralove, Czech Republic). Twelve SNPs were then determined by direct sequencing. PCR products were generated using 50 ng of genomic DNA in a 25 µl final volume containing 2.5 µl of 10× reaction buffer consisting of 0.8 (region 1) or 1.6 mM $MgCl_2$ (regions 2–4), 0.25 mM dNTPs, 0.2 µM of each primer, and 0.5 µl of *Taq* DNA polymerase, 1 U/µl (all chemicals except for dNTPs from Top-Bio, Vestec, Czech Republic). Primer sequences and optimized conditions for PCR cycling are specified in **Table S3 in File S1**. The PCR products were resolved and analyzed on 2% agarose gel containing ethidium bromide and visualized by ultraviolet light. All samples containing the PCR products were then sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) with 5 ng of PCR product and 2 pmol of universal M13 sequencing primer in a 10 µl final reaction volume. PCR conditions for sequencing reactions were as recommended by the producer (Life Technologies). Separate sequencing reaction included a control template pGEM-3Zf(+) under the same conditions as above. Sequencing products were purified by EDTA/sodium acetate/ethanol precipitation. DNA sequencing was performed on Applied Biosystems 3130×L Genetic Analyzer and the results were evaluated by Sequencing Analysis Software v5.2 (Life Technologies). About 10% of samples were re-sequenced with 100% conformity of the results.

### ABCC1 gene expression

Total RNA was isolated from frozen tissues, stored, and characterized as described [21]. cDNA was synthesized using 0.5 µg of total RNA and random hexamer primers with the help of RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Quality of cDNA in terms of DNA contamination was confirmed by PCR amplification of *ubiquitin C* [26]. Quantitative real-time PCR (qPCR) of ABCC1 and reference genes EIF2B1 (OMIM:606686), MRPL19 (OMIM:611832), IPO8 (OMIM:605600), and UBB (OMIM:191339) was performed in RotorGene 6000 (Corbett Research, Sydney, Australia) as described [13]. Reference genes for data normalization were selected using software programs geNorm (version 3.5) and NormFinder (version 19) (see File S1). The qPCR study design adhered to the MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) [27].

### Statistical analyses

The following differences in distribution of genotypes were evaluated: wild type *vs*. minor allele carrier (dominant model) and rare genotype carrier *vs*. wild type allele carrier (recessive model). The additive model was also tested. Haplotypes were evaluated using HaploView software program version 4.2 [24]; phasing of haplotypes prior to a block selection was done using the E-M algorithm and the block selection was based on confidence intervals [28]. Associations between categorized values as genotypes or haplotypes and clinical-pathological data were analyzed using the two-sided Fisher's Exact test. Clinical and pathological

**Table 3.** Distribution of *ABCC1* diplotypes predicted by HaploView v4.2.

| **Diplotype 1** | | **rs11866794** | | |
|---|---|---|---|---|
| | | **GG** | **GC** | **CC** |
| rs35625 | TT | **215** | 1 | 1 |
| | TC | **161** | **73** | 0 |
| | CC | 39 | 34 | 7 |
| **Diplotype 2** | | | rs35626 | |
| | | GG | GT | TT |
| rs4148351 | CC | **258** | **140** | 17 |
| | CT | 2 | **80** | 14 |
| | TT | 1 | 0 | 9 |
| **Diplotype 3** | | | rs3851711 | |
| | | TT | TC | CC |
| rs3888565 | GG | **151** | **163** | **66** |
| | GA | 0 | **81** | **53** |
| | AA | 0 | 4 | 20 |

Numbers of patients with combinations of diplotypes presented.
The most frequent diplotypes used for statistical analyses in bold.
doi:10.1371/journal.pone.0101740.t003

variables included menopausal status (pre- *vs*. post- or perimenopausal), stage (stage I *vs*. stage II–IV), tumor size (pT1 *vs*. pT2-4), lymph node metastasis (pN0 *vs*. pN1-3), histological type (invasive ductal *vs*. other invasive carcinoma) and grade (grade 1 *vs*. grade 2 or 3), expression of ER, PR, and HER2 (negative *vs*. positive), p53 expression (negative *vs*. positive), and molecular subtypes (triple negative *vs*. other and luminal A *vs*. luminal B/HER2−). Differences in transcript levels or age between patients divided by categorized data as genotypes, haplotypes, and clinical-pathological data were evaluated by nonparametric tests (Mann-Whitney, Kruskal-Wallis). Disease-free survival (DFS) was evaluated by the Kaplan-Meier method and the Breslow test was used for evaluation of the compared groups of patients. Multiparametric analysis was then performed by the Cox proportional hazards model. DFS was defined as the time elapsed between surgical treatment and disease progression or death from any cause. Patients lost to follow-up and patients with stage IV disease were excluded from DFS analyses. The results were evaluated by the statistical program SPSS v15.0 (SPSS, Chicago, IL). All p-values are departures from two-sided tests. A p-value of less than 0.05 was considered statistically significant. The correction for false discovery rate (FDR) was applied according to Benjamini and Hochberg [29] and q-values are provided for each comparison. The functional relevance of examined SNPs was analyzed *in silico* by Regulome DB (http://regulome.stanford.edu), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), and SIFT (http://sift.jcvi.org) programs. Genetic variants and their observed associations with clinical and functional phenotype were submitted to NCBI (The National Center for Biotechnology Information) ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar).

## Results

### Patients' characteristics

Clinical characteristics of patients are presented in **Table 1**.

### Distribution of genotypes and haplotypes

Twelve SNPs selected with the help of HaploView v4.2 pairwise tagging algorithm of the region including NBD1 and surrounding sequences of *ABCC1* were genotyped in 540 breast carcinoma patients. The rate of missing genotype data due to DNA of insufficient quality or quantity did not exceed 3.5% in particular SNPs.

The distribution of all analyzed SNPs (rs35623, rs4148351, rs35626, rs11075295, rs3851711, rs3888565, rs35625, rs4148350, rs35628, rs11866794, rs4148353, and rs4148356) is presented in **Table 2**. Genotype distribution of the studied SNPs did not significantly deviate from the Hardy-Weinberg equilibrium ($p > 0.01$). MAFs of these SNPs did not substantially differ from HapMap-CEU population ($n = 226$) available in dbSNP. Experimental data were reanalyzed by HaploView v4.2 and LD' values and haplotype blocks were predicted (**Figure 2**). This analysis revealed several SNP-SNP combinations (diplotypes, **Table 3**). To reach reasonable statistical power the most frequent diplotypes with $n > 40$ (highlighted in **Table 3**) were further analyzed.

### Associations of ABCC1 SNPs and diplotypes with transcript levels

The ABCC1 transcript level was previously assessed in tumors and non-neoplastic control tissues from breast cancer patients [13]. A subset of these patients with complete genotype data was included into this study ($n = 149$) and associations between genotypes, predicted diplotypes, and transcript levels were analyzed by Mann-Whitney or Kruskal-Wallis tests. Associations of all SNPs and frequent diplotypes with expression levels were analyzed but to retain concise style only significant results are reported (**Table 4** and **Figure 3**). Tumors from carriers of the wild type genotype in rs35623 or rs35628 expressed significantly lower ABCC1 transcript levels than those with the minor allele ($p = 0.003$ and $p = 0.004$, respectively; $q = 0.008$, both significant; **Table 4** and **Figures 3A, B**). A significant upward trend in the ABCC1 transcript level in the order CT-GT>CC-GT>CC-GG ($p = 0.023$; $q = 0.017$, non-significant; **Table 4** and **Figure 3C**)

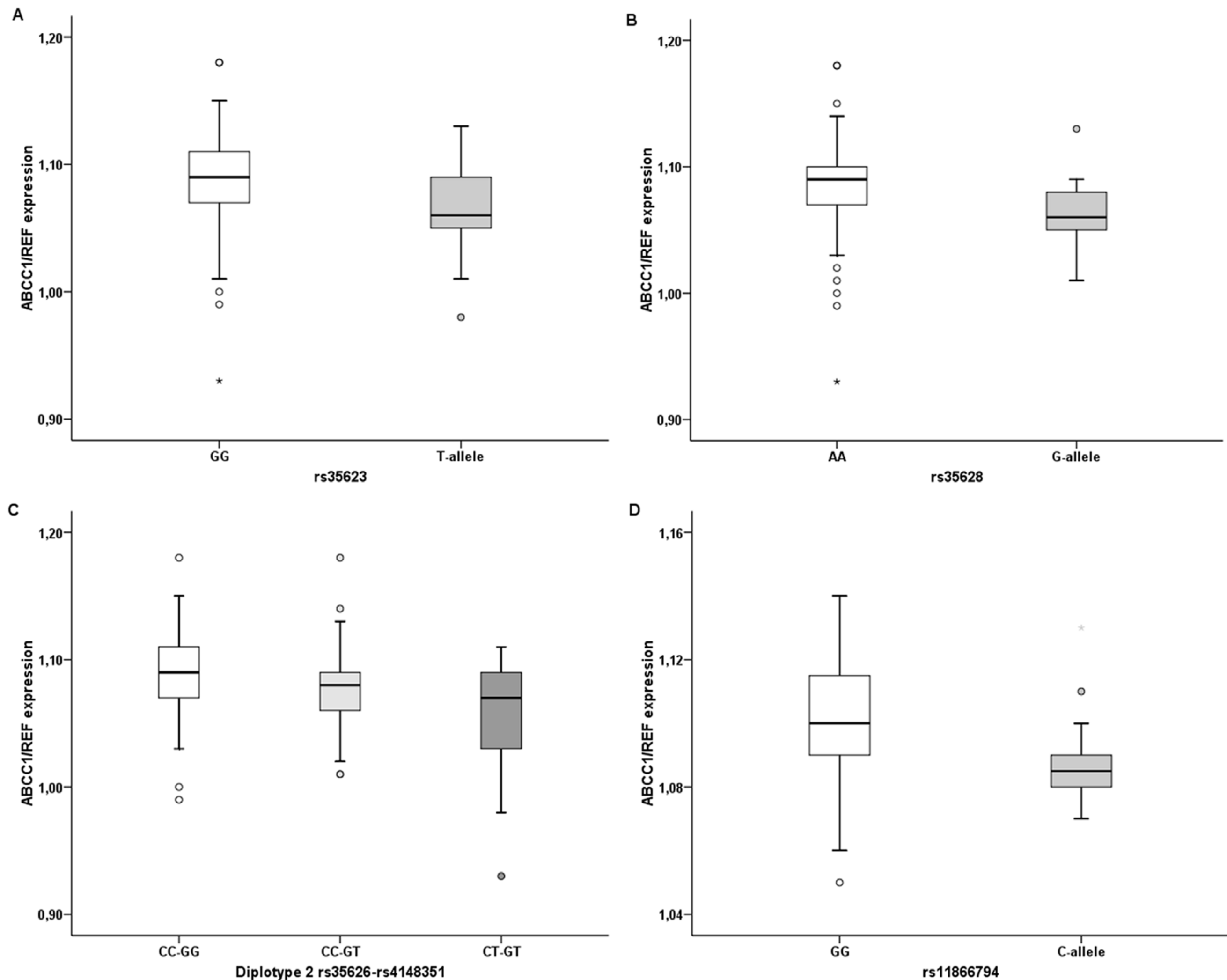


**Figure 3. Significant associations between transcript levels and polymorphisms in *ABCC1*.** All SNPs and frequent diplotypes were analyzed but to retain concise style only significant associations are reported.
doi:10.1371/journal.pone.0101740.g003

for the rs35626-rs4148351 diplotype was observed. Non-neoplastic control tissues from carriers of the wild type genotype in rs11866794 expressed lower ABCC1 transcript levels than those with the minor allele ($p = 0.017$; $q = 0.004$, non-significant; **Table 4** and **Figure 3D**). The rs4148356 SNP was predicted to be benign with a score of 0.014 by PolyPhen-2 and tolerated with a score 0.30 by SIFT programs. From synonymous SNPs, rs35626 was classified as likely to affect binding and linked to expression of a gene target (score 1f), rs35625, and rs11866794 as likely to affect binding (2c) by the Regulome DB program. The rest of SNPs was classified as having minimal binding evidence (4–6; **Table S4 in File S1**).

## Associations between clinical characteristics, therapy outcome, and ABCC1 SNPs and diplotypes

Associations of all SNPs and frequent diplotypes with clinical data were analyzed but to retain concise style only significant results are reported (**Table 5**). The *ABCC1* SNP rs3888565 was significantly associated with expression of estrogen receptor (**Table 5**). Carriers of the AA genotype had more frequently tumors without ER expression than carriers of the G allele ($p = 0.003$; $q = 0.004$, significant). Moreover, G allele in this SNP was associated with triple-negative disease exhibiting the worst prognosis of all molecular subtypes of breast carcinoma ($p = 0.008$; $q = 0.004$, non-significant). Regarding rs4148350, patients with stages II–IV (advanced disease) or lymph nodes affected by metastasis had a greater incidence of the T allele than those with early stage I or metastasis-free lymph nodes ($p = 0.005$ and $p = 0.028$, respectively; $q = 0.004$ and $q = 0.008$, both non-significant). Similarly, patients with HER2-positive tumors carried more frequently the T allele in rs4148350 than those without HER2 expression ($p = 0.014$; $q = 0.004$, non-significant). The T allele in rs4148353 also predisposed patients to tumors with ER expression in comparison with wild type carriers ($p = 0.049$; $q = 0.004$, non-significant). On the other hand, tumors of the T allele carriers in respect to rs4148353 were usually HER2-negative ($p = 0.001$; $q = 0.004$, significant; **Table 5**). Advanced stages II–IV, similarly as tumors with grades 2 or 3 occurred more frequently in carriers of the C allele in rs35625 than in those with the wild type TT ($p = 0.040$, $p = 0.029$, respectively; $q = 0.004$ and $q = 0.008$, both non-significant). Carriers of the C allele in rs3851711 had more frequently tumors of histological type other than ductal and exhibited more frequently triple-negative molec-

**Table 4.** Significant associations of *ABCC1* polymorphisms with expression levels.

| Genotype | n | Normalized ABCC1 expression in tumors (Mean Rank)* |
|---|---|---|
| **rs35623** | | |
| GG | 116 | 78.1 |
| GT or TT | 29 | 52.5 |
| Missing | 4 | – |
| p-value | | 0.003# |
| **rs35628** | | |
| AA | 117 | 73.6 |
| AG or GG | 21 | 46.8 |
| Missing | 11 | – |
| p-value | | 0.004# |
| **Diplotype 2 rs35626-rs4148351** | | |
| CC-GG | 66 | 69.3 |
| CC-GT | 38 | 60.0 |
| CT-GT | 20 | 44.7 |
| Missing | 25 | – |
| p-value | | 0.023 |
| **Genotype** | **n** | **Normalized ABCC1 expression in controls (Mean Rank)*** |
| **rs11866794** | | |
| GG | 48 | 36.9 |
| GC or CC | 18 | 24.4 |
| Missing | 1 | – |
| p-value | | 0.017 |

All SNPs and frequent diplotypes were analyzed but to retain concise style only significant associations are reported.
Footnotes:
*Analyzed by Mann-Whitney test. The higher is the rank the lower is the normalized expression ABCC1/reference genes.
#Result passed FDR analysis for multiple testing [29].
doi:10.1371/journal.pone.0101740.t004

ular subtype histology than those with the TT genotype (p = 0.040 and p = 0.039, respectively; q = 0.004 and q = 0.008, both non-significant).

No association between age at diagnosis, menopausal status, tumor size, expression of progesterone receptor, and p53 and the SNPs followed was found (results not shown). Large tumor size (pT2-4), presence of lymph node metastasis (pN1-3), lack of expression of hormonal receptors (ER and PR), and triple-negative molecular subtype were significant predictors of poor prognosis, i.e. short DFS in the set of chemotherapy-treated patients (p< 0.001, p = 0.001, p = 0.011, p = 0.001, and p = 0.003, respectively). Large tumor size (pT2-4), presence of lymph node metastasis (pN1-3), and lack of expression of PR were significant predictors of poor prognosis, i.e. short DFS in the set of hormonal therapy-treated patients (p = 0.001, p<0.001, and p = 0.031, respectively). Chemotherapy-treated patients carrying T allele in the rs4148353 SNP had longer DFS than those with wild type GG genotype in univariate analysis (n = 271, p = 0.043; **Figure 4A**). On the other hand, hormonal therapy-treated patients with the wild type AA genotype in the rs35628 had longer DFS than patients carrying the G allele (n = 353, p = 0.012; **Figure 4B**). Multivariate analysis using the Cox regression hazard model with pT, pN, ER, and PR expression, triple-negative molecular subtype, and individual SNPs has not confirmed association with DFS for rs4148353 (n = 252, p = 0.116). However, for rs35628 the association observed in univariate model remained significant in multivariate model with pT, pN, and PR expression (n = 323, p = 0.008). Survival analysis was not corrected for multiple testing.

## Discussion

Multidrug resistance frequently causes cancer treatment failure. Numerous *in vitro* and *in vivo* data revealed that multidrug resistance is often due to enhanced expression ABC transporters [30]. Thus, in depth analysis of ABC transporters appears inevitable for individualization of treatment.

The multidrug resistance-associated protein 1 encoded by the *ABCC1* gene is one of the most studied ABC transporters. Very recently, we demonstrated significant overexpression of ABCC1 transcript in tumors compared to adjacent non-neoplastic tissues from breast cancer patients and suggested its intratumoral levels as potential modifiers of breast carcinoma progression [13]. Another contemporary study has suggested that a high ABCC1 protein expression is a negative prognostic marker, as it has been found in highly aggressive molecular subtypes of breast carcinoma [12]. Despite already accumulated knowledge on ABCC1 there are significant gaps in understanding its role in cancer therapy and prognosis which preclude clinical applications.

NBD1 of ABCC1 contains several functional motifs, ATP-binding site, Walker A/P-loop, Q-loop/lid, ABC transporter signature, Walker B, D-loop, and H-loop/switch (**Figure 1**). Unlike most ABCC proteins, NBD1 of ABCC1 binds ATP with

**Table 5.** Significant associations of *ABCC1* polymorphisms with clinical data.

| Characteristics | rs3888565 | | p-value* |
|---|---|---|---|
| | GG/GA | AA | |
| ER negative | 119 | 13 | |
| ER positive | 380 | 10 | 0.003[#] |
| Triple negative | 49 | 7 | |
| Other subtype | 465 | 17 | 0.008 |
| **Characteristics** | **rs4148350** | | **p-value*** |
| | **GG** | **GT/TT** | |
| Stage II–IV | 229 | 40 | |
| Stage I | 199 | 14 | 0.005 |
| pN1-3 | 162 | 29 | |
| pN0 | 276 | 26 | 0.028 |
| HER2 negative | 315 | 29 | |
| HER2 positive | 96 | 20 | 0.014 |
| **Characteristics** | **rs4148353** | | **p-value*** |
| | **GG** | **GT/TT** | |
| ER negative | 111 | 16 | |
| ER positive | 304 | 78 | 0.049 |
| HER2 negative | 270 | 76 | |
| HER2 positive | 106 | 10 | 0.001[#] |
| **Characteristics** | **rs35625** | | **p-value*** |
| | **TT** | **TC/CC** | |
| Grade 2 or 3 | 133 | 222 | |
| Grade 1 | 51 | 51 | 0.029 |
| Stage II–IV | 101 | 46 | |
| Stage I | 102 | 26 | 0.040 |
| **Characteristics** | **rs3851711** | | **p-value*** |
| | **TT** | **TC/CC** | |
| Ductal type | 121 | 277 | |
| Other type | 26 | 100 | 0.040 |
| Triple-negative | 11 | 21 | |
| Other subtype | 140 | 118 | 0.039 |

All SNPs and frequent diplotypes were analyzed but to retain concise style only significant associations are reported.
Footnotes:
*Analyzed by two-sided Fisher's Exact test.
[#]Result passed FDR analysis for multiple testing [29].
doi:10.1371/journal.pone.0101740.t005

high affinity but has low hydrolytic activity, while the reverse is true of NBD2 [31]. Besides this functional asymmetry, it seems obvious that NBD1 and NBD2 cooperate together and with surrounding transmembrane cytoplasmic loops may influence substrate selectivity and the proper assembly and trafficking of ABCC1 to the plasma membrane [32,33]. Moreover, very recently, virtual screening of X-ray crystal structure of ABCC1 NBD1 [34] revealed that about 5% of the National Cancer Institute compounds possessed lower docking scores than ATP in ABCC1 NBD1 and it has been suggested that the compounds identified may be potential inhibitors of ABCC1 and require further pharmacological analysis [35]. Apparently, the role of ABCC1 as predictive biomarker and potential drug target in human cancers raises further interest.

The present study addressed yet unexplored associations between genetic variability in NBD1 and adjacent sequences of *ABCC1* and clinical course of breast cancer. Further, it evaluated relations between genotype and phenotype represented by transcript levels in tissues of breast cancer patients.

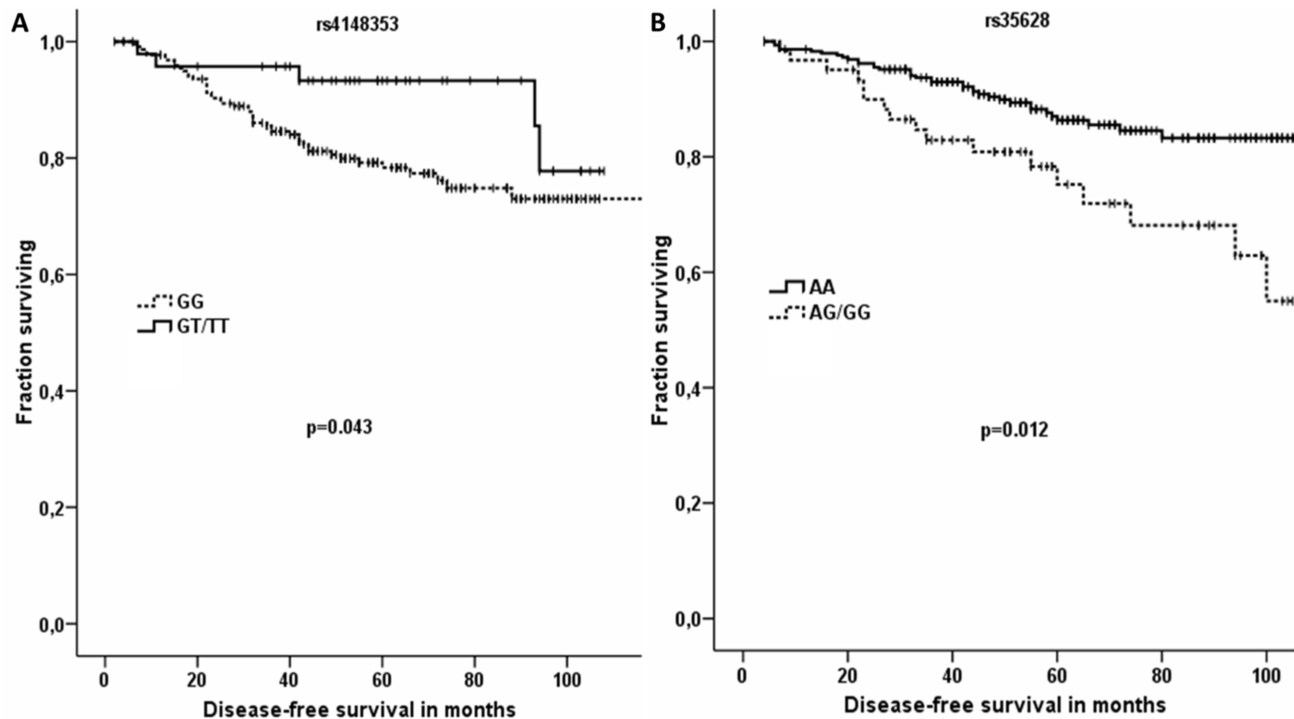


**Figure 4. Significant associations between DFS of patients with breast carcinoma and SNPs in *ABCC1*.** Kaplan-Meier survival curves for patients treated by chemotherapy (A) and hormonal therapy (B) were analyzed by Breslow test. In part A, dashed line represents DFS of patients with the GG genotype in rs4148353, while solid line indicates that of patients with the T allele. In part B, dashed line represents DFS of patients with the G allele in rs35628 and solid line DFS of those with the AA genotype. All SNPs have been analyzed but to retain concise style only significant associations are reported.
doi:10.1371/journal.pone.0101740.g004

Analogously with the ABCB1/P-glycoprotein [36], in the present study, we have observed associations between genetic variability in *ABCC1* and its transcript levels in tissues from breast cancer patients. Carriers of the wild type genotype in rs35623 or rs35628 SNPs had significantly lower ABCC1 levels in their tumors than the rest of patients, suggesting their potential as predictors of treatment outcomes. The association of the rare CT-GT diplotype rs35626-rs4148351 with ABCC1 transcript levels observed in the uncorrected analysis has not passed the FDR test and should be replicated on a larger sample size.

The analysis of associations between *ABCC1* SNPs and transcript levels could be confounded by the fact that some patients received pre-operative chemotherapy. Some ABC transporters may be induced by chemotherapy [37]. However, in our study the difference in ABCC1 transcript levels between post- and pre-treatment patients was non-significant ($p>0.05$).

Most interestingly, the rs35628 SNP significantly influenced DFS of patients treated by hormonal therapy in both univariate and multivariate analysis. Taken together, patients with the wild type genotype AA in rs35628 SNP had lower ABCC1 levels in tumors and better survival rates after hormonal therapy than those with the G allele. Cell lines with overexpression of ABCC1 are resistant to anticancer drugs [30] and high expression of ABCC1 protein was associated with shorter DFS [38]. The role of ABCC1 in the efflux of anticancer drugs has been recently proposed [39,40]. On the other hand, the intratumoral ABCC1 transcript level did not modify DFS of unselected patients ($n=88$) or patients stratified according to the therapy type, referring to a more complex phenomenon [13]. We also have not found significant association between transcript and protein levels of ABCC1 ($n=30$) in the previous study on independent set of patients [13].

Tamoxifen metabolites endoxifen and 4-hydroxy-tamoxifen are substrates of the ABCB1 transporter *in vitro* [41], but the association of genetic variation in *ABCB1* and tamoxifen effectiveness is unknown. The role of ABCC1/MRP1 in the transport of tamoxifen, its metabolites or aromatase inhibitors also remains vastly unexplored [42] and thus it is currently impossible to draw any conclusive remarks from our observations in hormonally-treated breast cancer patients.

We have also found out that chemotherapy-treated carriers of the T allele in rs4148353 SNP had significantly better DFS than those with the wild type GG genotype. However, this association has not been confirmed by multivariate analysis. The association of rs4148353 with DFS could be modulated by the significant associations of this SNP with ER and mainly HER2 which were shown to be the best predictors of chemotherapy response in breast carcinoma [43]. Patients carrying the T allele had higher frequency of ER-positive or HER2-negative tumors when compared with wild type carriers in the present study and the lack of expression of hormonal receptors or the triple-negative molecular subtype of breast cancer were indeed significant predictors of poor DFS.

Despite the fact that the *ABCC1* rs4148356 SNP located between the Walker A and B motifs in NBD1 has been shown to affect resistance to a number of anticancer drugs [20], we did not find association of this SNP with DFS in breast cancer patients. We confirmed the previously observed lack of effect of rs4148356 (R723Q, 2168G>A) on ABCC1 expression [44]. Also *in silico* analyses performed by PolyPhen-2 [45] and SIFT [46] programs

support our observations. Ten other non-synonymous SNPs leading to amino acid substitutions (Cys43Ser (G128C, rs41395947), Thr73Ile (C218T, rs41494447), Ser92Phe (C257T, rs8187844), Thr117Met (C350T, no rs number available), Arg230Gln (G689A, rs8187848), Arg633Gln (G1898A, rs112282109), Ala989Thr (G2965A, rs35529209), Cys1047Ser (G3140C, rs13337489), Arg1058Gln (G3173A, rs41410450), and Ser1512Leu (C4535T, rs369410659)) followed earlier had no effect on ABCC1 expression either, indicating that single amino acid substitutions may not necessarily influence the activity of the final protein [44]. No significant effect of the synonymous SNPs G816A (rs2230669), T825C (rs246221), T1684C (rs35605), and G4002A (rs2230671) on ABCC1 transcript level in peripheral CD4+ cells has been observed as well [15]. From the synonymous SNPs followed by the present study, *in silico* analyses by help of Regulome DB [47] suggested that rs35625, rs35626, and rs11866794 likely affect regulation of target gene transcription.

The lack of validation study in independent sample set may be seen as limitation of the present study. By searching Catalog of Published Genome-Wide Association Studies at NHGRI (www.genome.gov) and GWAS Central (www.gwascentral.org) we have found no supportive data for associations between *ABCC1* SNPs with breast carcinoma survival or therapy response that could support our results. Microarray study that explored associations of transcript levels with SNP markers from the International HapMap Project in lymphoblastoid cells of 57 unrelated CEPH individuals has not observed such association(s) for ABCC1 [48].

Significant associations of non-coding SNPs with expression and clinical phenotype observed by the present study were not confirmed by *in silico* analyses or additional experimental data. This fact limits the interpretation of the results before complex functional study is completed.

In conclusion, according to our present data, SNPs rs35623 and rs35628 in non-coding regions around NBD1 may modulate ABCC1 transcript levels in breast tumors, thus contributing to a complex pattern of chemotherapy resistance by so far unknown mechanism. Associations of rs35628 and rs4148353 with DFS of breast cancer patients warrant further studies aimed at validation or disqualification of these putative prognostic markers.

## Supporting Information

**File S1** Contains the following files: **Material and Methods S1. References S1. Table S1:** Chemotherapy and hormonal therapy regimens. **Table S2:** Positions of the analyzed SNPs in *ABCC1*. **Table S3:** Sequencing primers and PCR conditions for assessment of polymorphisms in NBD1 of *ABCC1*. **Table S4:** *In silico* analysis of functional significance of all studied polymorphisms in NBD1 of *ABCC1*. **Figure S1:** Flow diagram of the study.
(DOC)

## Author Contributions

Conceived and designed the experiments: RV DV VP PS. Performed the experiments: TK VH RV. Analyzed the data: TK KR MT KK MA LV PS. Contributed reagents/materials/analysis tools: VP KR MT KK MA LV PV. Wrote the paper: TK RV VH DV PV PS.

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*Supporting Information*

# Non-coding polymorphisms in nucleotide binding domain 1 in *ABCC1* gene associate with transcript level and survival of patients with breast cancer


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## Supplementary Material and Methods

*Patient samples*

After histopathological processing, tissue specimens were snap-frozen in liquid nitrogen. Since this point the pathological processing of the samples differed. In Motol, five-micrometer cryostat sections were prepared for isolation of total RNA. The presence of tumor cells in the sample was histologically verified in the first and in the last section of a row. The sections cut in the layers between the aforementioned histological controls were used for total RNA isolations. Specimens collected in Institute for the Care for Mother and Child were transferred to Biolab for pathological processing. Two specimens, the macroscopically apparent tumorous tissue and the non-tumorous mammary tissue in minimum distance of 20 mm from the tumor, both of 4-5 mm in diameter, were excised from the native tissue sample. One cryostat section from each block was stained by hematoxylin and eosin to confirm the content of tumor cells in both types of specimens. The frozen blocks of tissue were transported in dry ice to the main investigator laboratory for RNA isolation. In both pathology laboratories, the standard processing of surgical sample and diagnostic histological evaluation was followed according to WHO classification [1]. The following data on patients were retrieved from medical records: age, menopausal status, date of diagnosis of breast cancer, personal and family anamnesis (number of relatives affected by breast cancer, ovarian cancer or other malignant diseases), tumor size, lymph node metastasis, clinical stage, histological type and grade of tumor, expression of estrogen, progesterone receptors, and HER-2, expression of p53 protein, therapy, response (in neoadjuvant set only) and disease-free survival.

*Immunohistochemical detection of p53 protein expression*

Fresh tissue samples of the mammary tumors were fixed in standard neutral buffered 4% formaldehyde for up to 26 hours and embedded into paraffin with classical histological techniques. For immunohistochemical investigation 3 μm thick histological sections were utilized. Primary antibody against the p53 (clone DO-7; monoclonal mouse antibody detecting both mutant and wild type p53 protein) was purchased from Dako (Dako, Glostrup, Denmark). Antibodies were diluted with Dako Antibody diluent (1:50). For p53 detection, the sections were further processed with heat-induced epitope retrieval in 10 mmol/l citrate buffer pH 6.0 in water bath (40 min heating at 95-99° C and then 20 min cooling at room temperature). Tissues were incubated with primary antibodies overnight at 4° C. Detection was performed with peroxidase/diaminobenzidine system. Evaluation of binding of both primary antibodies was performed with Dako REAL Detection System (LSAB+, biotinylated secondary goat anti-mouse antibodies/streptavidin conjugated to horseradish peroxidase). As a chromogen, 0.04 % DAB (3,3´-diaminobenzidine tetrahydrochloride dihydrate; Fluka, Buchs, Switzerland) in 50 mmol/l TRIS (Tris-hydroxymethyl amino methane)/0.015 % $H_2O_2$ was used. Several p53 positive cells were present in each sample analyzed. p53 status was evaluated as positive, if more than 50% of tumor cells were immunohistochemically stained according to the previously published evaluation procedure [2, 3].

*Selection of reference genes*

For selection of reference genes, 96-well TaqMan Array Plates (Life Technologies) were used and evaluated as previously published [4, 5]. EIF2B1, MRPL19, IPO8, and UBB were selected as the most stable reference genes for data normalization.

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**Supporting Information Table S1:** Chemotherapy and hormonal therapy regimens

| Characteristics | Type | n | % |
|---|---|---|---|
| **Neoadjuvant regimen (n=122)** | Anthracycline in combination | 112 | 91.8 |
| | Taxane alone | 3 | 2.5 |
| | Hormonal therapy | 5 | 4.1 |
| | CMF only* | 2 | 1.6 |
| **Adjuvant or palliative regimen (n=251)** | Anthracycline in combination | 177 | 70.5 |
| | Taxane alone | 54 | 21.5 |
| | CMF only* | 20 | 8.0 |
| **Hormonal regimen (n=395)** | Tamoxifen only | 184 | 46.6 |
| | Aromatase inhibitors only | 105 | 26.6 |
| | Tamoxifen & aromatase inhibitors | 106 | 26.8 |

Footnotes:

*CMF=cyclophosphamide/methotrexate/5-fluorouracil combination

**Supporting Information Table S2:** Positions of the analyzed SNPs in *ABCC1*

| SNP | Position (Chr16) | Alleles captured by analysis |
|---|---|---|
| rs35623 | 16076966 | rs35621, rs35623, rs35628, rs35629, rs152029, rs152030 |
| rs35625 | 16077067 | rs35625 |
| rs11866794 | 16077075 | rs11866794 |
| rs4148350 | 16077978 | rs4148350 |
| rs4148351 | 16078069 | rs4148351, rs4148355 |
| rs35626 | 16078116 | rs2074086, rs35626, rs152028 |
| rs35628 | 16078607 | rs35628 |
| rs4148353 | 16078649 | rs4148353 |
| rs4148356 | 16084776 | rs4148356 |
| rs11075295 | 16085188 | rs11075295, rs7185286 |
| rs3888565 | 16090546 | rs3888565 |
| rs3851711 | 16090588 | rs4148354, rs3851711 |

**Supporting Information Table S3:** Sequencing primers and PCR conditions for assessment of polymorphisms in NBD1 of *ABCC1*

| Region | PCR conditions | Primer sequences 5´ → 3´* | Product size | $Mg^{2+}$concentration |
|---|---|---|---|---|
| **1** | initial hold 5´at 94°C<br>35 cycles: 0:30 at 94°C, 0:30 at **65**°C and 0:30 at 72°C<br>final hold 5´ at 72°C | F tgtaaaacgacggccagttgcacatcctgtagtcccagtt<br>R caggaaacagctatgaccacatgcaaacctctctccactg | 420 bp | 0.8 mM |
| **2** | initial hold 5´at 94°C<br>35 cycles: 0:30 at 94°C and 0:30 at **68**°C<br>final hold 5´ at 72°C | F tgtaaaacgacggccagttccctctctgtgaccttgaaca<br>R caggaaacagctatgaccacaattgaagcaggcaggattt | 897 bp | 1.6 mM |
| **3** | initial hold 5´at 94°C<br>35 cycles: 0:30 at 94°C, 0:30 at **63**°C and 0:30 at 72°C<br>final hold 5´ at 72°C | F tgtaaaacgacggccagtccctcttgccaaagcaatagtt<br>R caggaaacagctatgaccgcagtcatgtgaccacaaaggt | 725 bp | 1.6 mM |
| **4** | 3´ at 94°C<br>10 cycles: 0:30 at 94°C, 0:30 at **65 to 55**°C (touch down) and 0:30 at 72°C and 25 cycles: 0:30 at 94°C, 0:30 at **65**°C and 0:30 at 72°C<br>final hold 5´ at 72°C | F cgcacgtgtcctgttcttta<br>R catcatgttgtccaggctca | 371 bp | 1.6 mM |

The whole NBD1 and surrounding sequence was divided into four regions for SNP analysis. These regions were then amplified by PCR and sequenced.

Footnotes:
* F – forward, R – reverse primer, M13 sequence adaptors used for sequencing underlined, for sequencing of the region 4 unmodified primers were used.

**Supporting Information Table S4:** *In silico* analysis of functional significance of all studied polymorphisms in NBD1 of *ABCC1*

The functional significance of examined SNPs was analyzed *in silico* by Regulome DB (http://regulome.stanford.edu), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and SIFT (http://sift.jcvi.org).

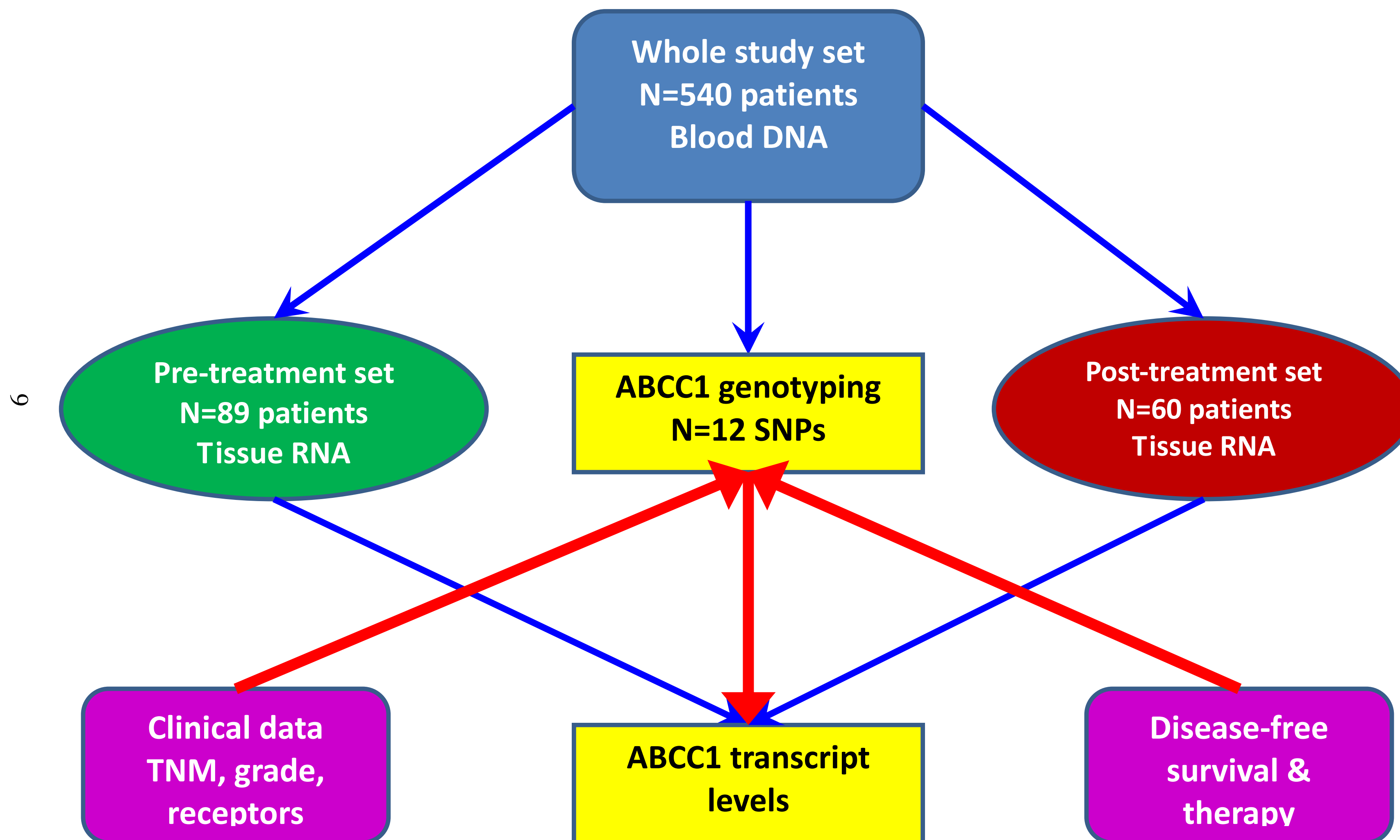
| **SNP position** | **SNP** | **Result of *in silico* analysis by Regulome DB** |
|---|---|---|
| chr16:16169464 | rs35623 | TF binding or DNase peak (5) |
| chr16:16169565 | rs35625 | TF binding + matched TF motif + DNase peak (2c) |
| chr16:16169573 | rs11866794 | TF binding + matched TF motif + DNase peak (2c) |
| chr16:16170476 | rs4148350 | TF binding or DNase peak (5) |
| chr16:16170567 | rs4148351 | No Data |
| chr16:16170614 | rs35626 | eQTL + TF binding / DNase peak (1f) |
| chr16:16171105 | rs35628 | Minimal binding evidence (6) |
| chr16:16171147 | rs4148353 | No Data |
| chr16:16177274 | rs4148356 | No Data |
| chr16:16177686 | rs11075295 | TF binding + DNase peak (4) |
| chr16:16183044 | rs3888565 | TF binding or DNase peak (5) |
| chr16:16183086 | rs3851711 | TF binding or DNase peak (5) |

Footnote: TF = transcription factor, eQTL = expression quantitative trait loci

The rs4148356 SNP was predicted to be benign with a score of 0.014 by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and tolerated with a score 0.30 by SIFT (http://sift.jcvi.org) programs.

**Supporting Figure S1: Flow diagram of the study**

Selection process and laboratory analyses are displayed by blue lines and statistical analyses by red lines.



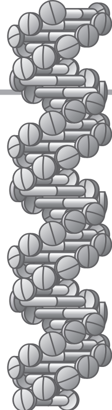
# Příloha č. 5:

**Hlaváč V**, Brynychová V, Václavíková R, Ehrlichová M, Vrána D, Pecha V, Koževnikovová R, Trnková M, Gatěk J, Kopperová D, Gut I, Souček P

**The expression profile of ATP-binding cassette transporter genes in breast carcinoma**

Pharmacogenomics. 2013;14(5):515-529 [IF: 3,857] 38 % *)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.



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# The expression profile of ATP-binding cassette transporter genes in breast carcinoma

**Aim:** ATP-binding cassette (ABC) transporters contribute to development of resistance to anticancer drugs via ATP-dependent drug efflux. A major goal of our study was to investigate associations between the expression of ABC transporters and outcome of breast carcinoma patients. **Patients & methods:** Transcript levels of all 49 human ABC transporters were determined in post-treatment tumor and non-neoplastic tissue samples from 68 breast carcinoma patients treated by neoadjuvant chemotherapy. Six ABC transporters were then evaluated in independent series of 100 pretreatment patients. **Results:** *ABCA5/6/8/9/10*, *ABCB1/5/11*, *ABCC6/9*, *ABCD2/4*, *ABCG5* and *ABCG8* were significantly downregulated and *ABCA2/3/7/12*, *ABCB2/3/8/9/10*, *ABCC1/4/5/10/11/12*, *ABCD1/3*, *ABCE1*, *ABCF1/2/3* and *ABCG1* were upregulated in post-treatment tumors compared with non-neoplastic tissues. Significant associations of intratumoral levels of *ABCC1* and *ABCC8* with grade and expression of hormonal receptors were found in both sets of patients. *ABCA12*, *ABCA13* and *ABCD2* levels were significantly associated with the response to neoadjuvant chemotherapy in post-treatment patients. Protein expression of ABCA12, ABCC8 and ABCD2 in tumor tissues of patients with breast carcinoma was observed by immunoblotting for the first time. **Conclusion:** *ABCA12*, *ABCA13*, *ABCC1*, *ABCC8* and *ABCD2* present potential modifiers of progression and response to the chemotherapy of breast carcinoma.

Original submitted 27 July 2012; Revision submitted 5 February 2013

**KEYWORDS: ABC transporters ▪ breast carcinoma ▪ expression ▪ prognosis ▪ resistance ▪ response**

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Breast cancer is the most common cancer in women with an estimated 1.38 million new cases worldwide in 2008 [1]. While survival is improving, the burden of this disease is predicted to increase globally [2]. The prognosis of breast cancer patients is closely associated with the response of the tumor cells to chemotherapy. Tumor cells may be resistant *per se* or develop multidrug resistance (MDR) to a variety of chemotherapy drugs. The reasons for MDR are not clear but have been attributed to alterations in many molecular pathways. Decreased uptake of water-soluble drugs, increased repair of DNA damage, reduced apoptosis and increased energy-dependent efflux of hydrophobic drugs exert influence on these pathways [3].

Drug efflux from tumor cells is most frequently associated with overexpression of one or more membrane-bound ATP-binding cassette (ABC) transporters [4]. A total of 49 human ABC transporters represent a large family of transporter proteins that translocate a wide variety of substrates, including metabolic products, lipids, sterols and drugs across extra- and intra-cellular membranes [5]. The *ABCB1* product P-gp represents the most extensively studied ABC transporter [6]. *ABCB1*/P-gp overexpression has been associated with a poor drug response in breast cancer patients [7,8], while other authors [9–11] found no association of *ABCB1*/P-gp expression with breast cancer prognosis. MRP1/*ABCC1* is involved in MDR of a variety of solid tumors. A strong association between ABCC1 expression level and reduced time to relapse and overall survival was revealed [12]. No association of MRP1/*ABCC1* expression with breast cancer prognosis has been described [4]. *ABCG2* encoding BCRP also belongs to the frequently studied ABC transporters [13]. The role of other ABC genes in MDR is much less understood. Transcript levels of several ABC transporters in breast tumors are correlated with the clinical response to neoadjuvant chemotherapy (NACT) with sequential weekly paclitaxel/FEC (5-fluorouracil plus epirubicin plus cyclophosphamide) [14].

It seems that the characterization of the expression profile of genes associated with MDR, prognosis and response to chemotherapy may be useful for individualization of cancer therapy. The aim of our study was to explore gene-expression levels of all members of the

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ABC transporter gene family in post-treatment tissues from NACT-treated breast cancer patients. Profiles were compared with clinical data and response of the patients to NACT in order to evaluate candidate biomarkers with prognostic and predictive value. Candidate biomarkers identified by the evaluation set were evaluated in an independent pretreatment set of breast cancer patients.

## Patients & methods

### Materials

Phenol, chloroform, RNase A, proteinase K, ultrapure agarose, and chemicals for preparation of buffers were purchased from Sigma-Aldrich (Prague, Czech Republic). Deoxynucleotides for PCR and molecular weight standard for electrophoresis (ϕX174DNA/*Hae*III digest) were products of New England Biolabs, Inc. (MA, USA). Taq-Purple DNA polymerase and Combi PPP Master Mix for PCR were supplied by Top-Bio s.r.o. (Prague, Czech Republic).

### Patients

Post-treatment tissue samples of human carcinomas of the mammary gland were prospectively obtained from 68 incident breast cancer patients diagnosed at the Department of Oncosurgery, Medicon (Prague, Czech Republic), during 2006–2010 (evaluation set, described in detail elsewhere [15]). Patients were treated with 5-fluorouracil-, anthracycline-, cyclophosphamide- and/or taxane-based NACT (**Supplementary Table 1**; www.futuremedicine.com/doi/suppl/10.2217/pgs.13.26). Paired adjacent tissue samples without morphological signs of carcinoma (nontumor controls) were available from 43 patients. The macroscopically apparent tumor tissue and the control tissue specimen at minimum distance of 20 mm from the tumor, both 4–5 mm in diameter, were excised from the native tissue sample. One cryostat section from each block was stained by hematoxylin and eosin to monitor the content of tumor cells in both types of specimens. Prospectively collected samples from 100 incident breast cancer patients diagnosed at the Faculty Hospital in Motol (Prague, Czech Republic) during 2003–2007 were used as the validation set. Paired adjacent tissue samples without morphological signs of carcinoma (nontumor controls) were available from 34 patients. Collection and handling of tissue samples was described elsewhere [16]. The following data on patients were retrieved from medical records: age, menopausal status, date of diagnosis with breast cancer, personal history, family history (number of relatives affected by breast cancer, ovarian cancer or other malignant diseases), tumor size, lymph node metastasis, clinical stage, histological type and grade of tumor, expression of ER, PR and HER2, expression of the Ki-67 protein (available in the evaluation set only), and NACT regimen and outcome. Expression of the p53 protein was available in the validation set. Expression of ER and PR was assessed according to the published procedure with the recommended 1% cutoff value [17]. HER2 status was defined as positive in samples with immunohistochemical score of 2+ or 3+ confirmed by FISH analysis [18]. Response to NACT was evaluated by Response Evaluation Criteria In Solid Tumors (RECIST) criteria as described [19]. For analysis of protein levels of candidate genes a training set was established. Pretreatment tumor tissue samples of human carcinomas of the mammary gland were prospectively obtained from 30 incident histologically verified breast cancer patients diagnosed at the Department of Surgery, Hospital Atlas (Zlin, Czech Republic) during 2012. Collection and handling of tissue samples and clinical data retrieval adhered to the above described design.

All patients were asked to read and sign an informed consent in agreement with requirements of the Ethical Commission of the National Institute of Public Health in Prague.

### Isolation of total RNA & cDNA preparation

Total RNA was isolated from frozen tissues using Trizol® reagent (Invitrogen, CA, USA). RNA quantity was assessed in duplicates by Quant-iT RiboGreen RNA Assay Kit (Invitrogen) using Infinite M200 multiplate reader (Tecan Group Ltd, Männedorf, Switzerland). RNA quality was assessed by measurement of RNA Integrity Number using Agilent RNA 6000 Nano Assay Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., CA, USA). The RNA integrity number was 5.4 on average with a range of 3.0–8.9. cDNA was synthesized using 0.5 µg of total RNA as previously described [16]. The cDNA from the evaluation set was then preamplified using 25 µl of TaqMan® PreAmp Master Mix (Life Technologies Corp., CA, USA), 12.5 µl of pooled assay mix containing all target TaqMan Gene Expression Assays (Life Technologies; listed in [20]; **Supplementary Table 2**), 5 µl of cDNA and nuclease-free water in a 50 µl final volume. A total of 14 preamplification cycles were used and the preamplification uniformity was evaluated according to the recommendation

of the manufacturer. cDNA from the validation set was used directly for quantification without preamplification procedure.

## Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed and results evaluated using RotorGene 6000 (Corbett Research, Sydney, Australia) for the evaluation set and ViiA™ 7 Real-Time PCR System (Life Technologies) for the validation set as described before [16,20]. Fragments spanning binding sites for TaqMan probe and primers for *ABCC1* (1202 bp), *ABCC8* (1695 bp) and *MRPL19* (883 bp) were subcloned into vector pDONR201 using Gateway™ Cloning Technology (Invitrogen) as previously described [16]. Absolute quantification with external standard curves generated from five decimal dilutions of the respective plasmid was performed. *ABCC1* and *ABCC8* transcript levels (target) were then normalized to levels of *MRPL19* as internal control ($copies_{target}/copies_{reference}$). Gene-expression and clinical data of the evaluation set were submitted to the Gene Expression Omnibus (GEO) repository under accession number GSE43807 [101].

## Selection of reference genes

For selection of reference genes, 96-well TaqMan Array Plates (Life Technologies) were used and evaluated as previously published [20,21]. *EIF2B1*, *MRPL19*, *IPO8* and *UBB* were selected as the most stable reference genes for data normalization.

The qPCR study design adhered to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines [22].

## Immunoblotting in human breast carcinoma tissues

Tumor tissue samples (n = 30) were stored at -80°C prior to protein isolation. Samples were grinded using a mortar and pestle and then protein and total RNA were isolated using AllPrep® DNA/RNA/Protein Mini Kit (Qiagen, Hildesheim, Germany) according to the manufacturer's protocol. Total RNA was then used for qPCR of *ABCA12*, *ABCC1*, *ABCC8* and *ABCD2* as described above. Protein concentration was determined by bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific Pierce Protein Research Products, IL, USA). Immunoblotting was performed as previously described [23]. Briefly, 20 µg of protein was used for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel) and transferred onto 0.2 µm Protran® nitrocellulose membrane (Whatman, Kent, UK). Firstly, membranes were blocked in 5% weight/volume nonfat dry milk (for *ABCC1* and *ABCD2*) or 5% weight/volume bovine serum albumin (for *ABCC8* and *ABCA12*) in Tris-buffered saline for 25 min at room temperature. Then, membranes were incubated with primary antibodies against *ABCA12* (dilution 1:1000; Aviva System Biology, CA, USA), *ABCC1* (dilution 1:50; Alexis Biochemicals, NY, USA), *ABCC8* (dilution 1:1000; Aviva System Biology), *ABCD2* (dilution 1:500; Abcam, Cambridge, UK) or β-actin (dilution 1:2000; Sigma-Aldrich) overnight at 4°C. Membranes were then incubated for 2 h at room temperature with corresponding horseradish peroxidase-conjugated secondary antibodies (dilution 1:6600; Santa Cruz Biotechnology, CA, USA). Protein bands were visualized with an enhanced chemiluminescence detection system (Thermo Scientific Pierce Protein Research Products) by Carestream Gel Logic 4000 PRO Imaging System (Carestream Health, CT, USA). Densitometry was performed using the Carestream v5.2 program (Carestream Health).

## Data analysis

Raw cycle threshold data were processed by REST2009 program (Qiagen). Each sample was assayed in duplicate and the mean value was used for calculations. Samples with higher cycle threshold than 40 were treated as missing data. For statistical analyses of associations of transcript levels with clinical data nonparametric tests (Kruskal–Wallis, Mann–Whitney U and Spearman's rank test) were used. Tested variables were as follows: age at diagnosis in years; menopausal status (premenopausal vs postmenopausal); tumor size in mm; lymph node metastasis (pN0 vs pN1–3); histological type; ductal versus other; pathological grade (G1 or G2 vs G3); stage (SI vs SII–SIV); ER, PR, HER2 and p53 expression (positive vs negative); expression of Ki-67 in percentage; and response to NACT (complete or partial pathological response vs stable disease or progression). Progression-free survival (PFS) was defined as the time elapsed between surgical treatment and disease progression or death from any cause [16]. Patients lost to follow-up (n = 11) and patients with stage IV disease (n = 1) were excluded from PFS analyses. Kaplan–Meier survival plots with the log-rank test were used for testing of PFS as previously published [20]. All p-values are departures from two-sided tests. A p-value of

**Table 1. Clinical characteristics of the followed groups of patients.**

| Characteristics | Evaluation set | Validation set |
|---|---|---|
| Age at diagnosis, mean ± SD (years) | 53.0 ± 11.5 | 60.0 ± 10.6 |
| Tumor size, mean ± SD (mm) | 21.0 ± 14.7 | 17.0 ± 11.2 |
| ***Menopausal status*** | | |
| Premenopausal | 31 (46) | 10 (10) |
| Postmenopausal | 37 (54) | 90 (90) |
| ***Lymph node metastasis*** | | |
| Absent (pN0) | 41 (60) | 54 (56) |
| Present (pN1–3) | 27 (40) | 42 (44) |
| Unknown | 0 (–) | 4 (–) |
| ***Pathological stage*** | | |
| I | 24 (37) | 36 (38) |
| II | 34 (52) | 41 (43) |
| III | 7 (11) | 18 (19) |
| IV | 0 (–) | 1 (1) |
| Not determined | 3 (–) | 4 (–) |
| ***Histological type*** | | |
| Invasive ductal carcinoma | 57 (84) | 83 (83) |
| Other type | 11 (16) | 17 (17) |
| ***Pathological grade*** | | |
| 1 | 8 (12) | 17 (17) |
| 2 | 29 (44) | 60 (62) |
| 3 | 29 (44) | 20 (21) |
| Unknown | 2 (–) | 3 (–) |
| ***ER status*** | | |
| Positive | 47 (69) | 68 (68) |
| Negative | 21 (31) | 32 (32) |
| ***PR status*** | | |
| Positive | 48 (71) | 59 (59) |
| Negative | 20 (29) | 41 (41) |
| ***Expression of HER2*** | | |
| Positive | 16 (24) | 26 (26) |
| Negative | 51 (76) | 74 (74) |
| Unknown | 1 (–) | 0 (–) |
| ***Expression of Ki-67*** | | |
| Mean ± SD | 32.6 ± 23.1 | Not available |
| Unknown | 1 (–) | – (–) |
| ***Expression of p53*** | | |
| Positive | Not available | 30 (31) |
| Negative | Not available | 66 (69) |
| Unknown | Not available | 4 (–) |

*Data are presented as number of patients (%), unless indicated otherwise.*
*SD: Standard deviation.*

**Table 1. Clinical characteristics of the followed groups of patients (cont.).**

| Characteristics | Evaluation set | Validation set |
|---|---|---|
| ***Response*** | | |
| Complete or partial response/ stable disease or progression | 38/25 (60/40) | Not applicable |
| Relapse/remission | Not applicable | 29/60 (33/67) |
| Not assessed | 5 (–) | 11 (–) |

*Data are presented as number of patients (%), unless indicated otherwise.*
*SD: Standard deviation.*

less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS v15.0 program (SPSS Inc., IL, USA).

## Results

Clinical characteristics of patients are described in Table 1.

### Differences in ABC transcript expression between tumors & non-neoplastic control tissues

*ABCA2*, *ABCA3*, *ABCA7*, *ABCA12*, *ABCB2*, *ABCB3*, *ABCB8*, *ABCB9*, *ABCB10*, *ABCC1*, *ABCC4*, *ABCC5*, *ABCC10*, *ABCC11*, *ABCC12*, *ABCD1*, *ABCD3*, *ABCE1*, *ABCF1*, *ABCF2*, *ABCF3* and *ABCG1* were significantly upregulated in tumors compared with control tissues from the evaluation set (n = 43 tissue pairs). *ABCA5*, *ABCA6*, *ABCA8*, *ABCA9*, *ABCA10*, *ABCB1*, *ABCB5*, *ABCB11*, *ABCC6*, *ABCC9*, *ABCD2*, *ABCD4*, *ABCG5* and *ABCG8* were significantly downregulated in tumors. No significant changes in expression of the rest of the ABC genes (*ABCA1*, *ABCA4*, *ABCA13*, *ABCB4*, *ABCB6*, *ABCB7*, *ABCC2*, *ABCC3*, *ABCC7*, *ABCC8*, *ABCC13*, *ABCG2* and *ABCG4*) between tumor and control tissues were found. The fold changes between tumor and control tissues with 95% CIs and p-values calculated by REST2009 are listed in Table 2.

### Associations of ABC transcript levels with clinical data in the evaluation set

Except for response to NACT, only levels of ABC transporters in tumors were evaluated. Tumor samples from two patients with complete pathological response were not evaluated owing to the absence of tumor cells.

A strong negative correlation of *ABCA10* and *ABCB1* levels with age was found (Spearman's ρ-correlation coefficient = -0.331 and -0.320, respectively; p = 0.006 and 0.008, respectively). Conversely, *ABCA12*, *ABCC11*, *ABCC12* and *ABCF1* positively correlated with age (ρ = 0.263, 0.255, 0.287 and 0.266, respectively; p = 0.030, 0.036, 0.021 and 0.028, respectively). Accordingly, premenopausal patients had significantly higher levels of *ABCA10* and *ABCB1* than postmenopausal patients (p = 0.022 and 0.026, respectively). Postmenopausal patients had significantly higher *ABCC12* levels than premenopausal patients (p = 0.030). Patients with stage I had significantly higher levels of *ABCB6* in tumors than patients with stages II–IV (p = 0.031).

Tumor size negatively correlated with *ABCB6* and *ABCB7* levels (Table 3). Patients without lymph node metastasis had significantly higher *ABCA2*, *ABCB4*, *ABCB5* and *ABCG4* levels than patients with affected nodes. *ABCA13*, *ABCB3* and *ABCC1* levels were significantly higher in patients with grade 3 (undifferentiated tumors) than in patients with grade 1 or 2 (well or moderately differentiated tumors). *ABCA2*, *ABCB7*, *ABCC8* and *ABCC11* levels were significantly higher in patients with grade 1 or 2 tumors. *ABCA13*, *ABCB3*, *ABCC1* and *ABCC10* levels were significantly higher in patients without expression of ER than in ER-positive patients. *ABCB7*, *ABCC8*, *ABCC11* and *ABCC13* levels were higher in tumors expressing ER. *ABCA13* and *ABCB6* levels were significantly higher in patients without expression of PR compared with PR-positive patients. *ABCA2*, *ABCA3*, *ABCC8*, *ABCC11* and *ABCC13* levels were significantly higher in patients with PR-expressing tumors. *ABCA13*, *ABCB2*, *ABCB3* and *ABCC1* levels positively, and *ABCC8* and *ABCC11* levels negatively correlated with the Ki-67 protein expression (Table 3). The most notable associations were found for *ABCC1* and *ABCC8* levels (association with grade, hormonal receptors and Ki-67 expression; Table 3).

Transcript levels of *ABCA13* and *ABCD2* in tumors and *ABCA12* in control tissues associated with the response to NACT. Patients with complete or partial response (responders) had significantly higher *ABCA13* and *ABCD2* levels than patients with stable or progressive disease (nonresponders; mean ± SD: 1.64 ± 0.17

**Table 2. Differences in expression of ATP-binding cassette transporters between tumor and control tissues in the evaluation set.**

| Gene symbol | p-value[†] | Tumor vs control | Expression difference (fold change) | 95% CI |
|---|---|---|---|---|
| *ABCA1* | NS | – | 0.86 | 0.20–7.16 |
| *ABCA2* | 0.002 | ↑ | 1.25 | 0.48–3.84 |
| *ABCA3* | <0.001 | ↑ | 1.61 | 0.38–9.65 |
| *ABCA4* | NS | – | 0.76 | 0.04–17.81 |
| *ABCA5* | <0.001 | ↓ | 0.51 | 0.11–1.79 |
| *ABCA6* | <0.001 | ↓ | 0.26 | 0.03–2.00 |
| *ABCA7* | <0.001 | ↑ | 1.70 | 0.43–7.25 |
| *ABCA8* | <0.001 | ↓ | 0.14 | 0.00–2.06 |
| *ABCA9* | <0.001 | ↓ | 0.23 | 0.02–1.58 |
| *ABCA10* | <0.001 | ↓ | 0.17 | 0.01–2.45 |
| *ABCA12*[‡] | <0.001 | ↑[§] | 4.48 | 0.01–2467.84 |
| *ABCA13*[‡] | NS | – | 0.64 | 0.01–284.79 |
| *ABCB1* | 0.015 | ↓ | 0.66 | 0.07–6.55 |
| *ABCB2* | <0.001 | ↑ | 1.81 | 0.42–12.06 |
| *ABCB3* | 0.009 | ↑ | 1.41 | 0.31–9.24 |
| *ABCB4* | NS | – | 1.20 | 0.23–6.64 |
| *ABCB5* | <0.001 | ↓ | 0.21 | 0.00–9.76 |
| *ABCB6* | NS | – | 1.03 | 0.41–3.31 |
| *ABCB7* | NS | – | 1.07 | 0.45–2.53 |
| *ABCB8* | <0.001 | ↑ | 1.75 | 0.42–7.82 |
| *ABCB9* | 0.003 | ↑ | 1.44 | 0.26–6.92 |
| *ABCB10* | <0.001 | ↑ | 1.26 | 0.50–2.75 |
| *ABCB11* | 0.030 | ↓ | 0.61 | 0.03–17.24 |
| *ABCC1*[‡] | <0.001 | ↑[§] | 1.37 | 0.52–3.74 |
| *ABCC2* | NS | – | 0.98 | 0.14–10.97 |
| *ABCC3* | NS | – | 1.27 | 0.13–17.57 |
| *ABCC4* | 0.001 | ↑ | 1.66 | 0.28–15.02 |
| *ABCC5* | <0.001 | ↑ | 1.69 | 0.33–8.64 |
| *ABCC6* | 0.001 | ↓ | 0.42 | 0.02–4.43 |
| *ABCC7* | NS | – | 0.94 | 0.01–243.99 |
| *ABCC8*[‡] | NS | –[§] | 0.80 | 0.00–73.31 |
| *ABCC9* | <0.001 | ↓ | 0.33 | 0.04–2.92 |
| *ABCC10* | <0.001 | ↑ | 1.38 | 0.50–4.09 |
| *ABCC11*[‡] | 0.019 | ↑ | 2.96 | 0.01–1104.83 |
| *ABCC12* | 0.014 | ↑ | 6.37 | 0.00–648,155.01 |
| *ABCC13* | NS | – | 1.04 | 0.00–1371.17 |
| *ABCD1* | 0.036 | ↑ | 1.24 | 0.30–5.18 |
| *ABCD2*[‡] | <0.001 | ↓[§] | 0.37 | 0.02–4.76 |

*[†]p-values calculated by REST2009 software (Qiagen, Hildesheim, Germany).*
*[‡]Genes used for validation study.*
*[§]Confirmed in the validation set (n = 100).*
*↑: Upregulation; ↓: Downregulation; NS: Nonsignificant.*

**Table 2. Differences in expression of ATP-binding cassette transporters between tumor and control tissues in the evaluation set (cont.).**

| Gene symbol | p-value† | Tumor vs control | Expression difference (fold change) | 95% CI |
|---|---|---|---|---|
| *ABCD3* | 0.003 | ↑ | 1.39 | 0.35–6.36 |
| *ABCD4* | 0.018 | ↓ | 0.86 | 0.34–1.91 |
| *ABCE1* | 0.031 | ↑ | 1.15 | 0.49–2.99 |
| *ABCF1* | <0.001 | ↑ | 1.39 | 0.52–3.94 |
| *ABCF2* | <0.001 | ↑ | 1.43 | 0.55–4.03 |
| *ABCF3* | <0.001 | ↑ | 1.29 | 0.52–3.14 |
| *ABCG1* | <0.001 | ↑ | 1.83 | 0.39–11.09 |
| *ABCG2* | NS | – | 0.74 | 0.06–7.48 |
| *ABCG4* | NS | – | 0.88 | 0.12–5.48 |
| *ABCG5* | 0.026 | ↓ | 0.40 | 0.00–673.52 |
| *ABCG8* | 0.020 | ↓ | 0.51 | 0.00–31.64 |

†p-values calculated by REST2009 software (Qiagen, Hildesheim, Germany).
‡Genes used for validation study.
§Confirmed in the validation set (n = 100).
↑: Upregulation; ↓: Downregulation; NS: Nonsignificant.

vs 1.73 ± 0.20; p = 0.048; and 1.30 ± 0.10 vs 1.36 ± 0.12; p = 0.024, respectively). The opposite was found for *ABCA12*, that is, nonresponders had significantly higher levels than responders (1.42 ± 0.19 vs 1.54 ± 0.14, respectively; p = 0.048). A weak association of *ABCC8* level with NACT outcome was observed too; nonresponders to NACT had higher *ABCC8* levels than responders (1.37 ± 0.15 vs 1.49 ± 0.23, respectively; p = 0.096). The rest of the genes did not significantly associate with the response (data not shown).

## Associations of ABC transcript levels with clinical data in the validation set

Genes strongly (p < 0.01) associated with the most important clinical data (as grade and hormonal receptors) and genes significantly associated with the response to NACT in the evaluation set were included into the validation study. Thus, *ABCA12*, *ABCA13*, *ABCC1*, *ABCC8*, *ABCC11* and *ABCD2* were selected for assessment in the validation set (n = 100) in order to confirm the results obtained with the evaluation set. *ABCA12*, *ABCA13*, *ABCC8*, *ABCC11* and *ABCD2* levels in tumors were significantly higher in the validation set when compared with the evaluation set (p < 0.001). *ABCC1* levels did not significantly differ between both sets (p = 0.057). *ABCC1* level in tumors negatively correlated with age (ρ = -0.201; p = 0.046). Accordingly, premenopausal patients had a significantly higher level of *ABCC1* than postmenopausal patients (p = 0.018). Conversely, *ABCC8* level in tumors positively correlated with age (ρ = 0.327; p = 0.001). Patients with stage I had higher levels of *ABCC1* and *ABCD2* in tumors than patients with stages II–IV (p = 0.018 and 0.010, respectively). However, these results were not observed in the evaluation set.

Associations with the rest of the tested clinical data are presented in Table 4. Significant associations of *ABCC1* and *ABCC8* levels in tumors with grade and expression of hormonal receptors observed in the evaluation set were replicated by the analysis of the validation set. A number of other associations was also observed; however, these associations were not found in the evaluation set (Tables 3 & 4).

All significant associations of *ABCC1* and *ABCC8* with clinical data obtained by relative quantification were confirmed also by the analysis of transcript levels of these two genes by absolute quantification (data not shown). None of the studied ABC transporters significantly associated with the PFS of patients divided to chemotherapy versus hormonal therapy subgroups in the validation set (Figure 1).

## Protein expression of ABCA12, ABCC1, ABCC8 & ABCD2 in breast tumors

Expression of ABCA12, ABCC1, ABCC8 and ABCD2 proteins was assessed by immunoblotting in protein lysates from breast tumor tissue samples and quantified by densitometry. ABCA13 was not assessed owing to a high molecular weight of this protein (predicted

**Table 3. Significant associations of ATP-binding cassette transporter transcript levels with clinical characteristics of breast cancer patients in the evaluation set.**

| Gene | Lymph node metastasis† | | Tumor size‡ (mm) | Grade† | | ER† | | PR† | | Ki-67 expression‡ |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Absent* | *Present* | | *1 or 2* | *3* | *Positive* | *Negative* | *Positive* | *Negative* | |
| *ABCA2* | 1.15 ± 0.05<br>p = 0.033 | 1.17 ± 0.04 | NS | 1.15 ± 0.04<br>p = 0.043 | 1.17 ± 0.05 | NS | NS | 1.15 ± 0.05<br>p = 0.017 | 1.18 ± 0.04 | NS |
| *ABCA3* | NS | NS | NS | NS | NS | NS | NS | 1.08 ± 0.08<br>p = 0.044 | 1.12 ± 0.05 | NS |
| *ABCA13*§ | NS | NS | NS | 1.75 ± 0.19<br>p = 0.020 | 1.63 ± 0.19 | 1.75 ± 0.17<br>p < 0.001 | 1.57 ± 0.19 | 1.74 ± 0.17<br>p = 0.004 | 1.58 ± 0.20 | ρ = 0.300<br>p = 0.014 |
| *ABCB2* | NS | NS | NS | NS | NS | NS | NS | NS | NS | ρ = 0.244<br>p = 0.047 |
| *ABCB3* | NS | NS | NS | 1.08 ± 0.06<br>p = 0.020 | 1.04 ± 0.08 | 1.08 ± 0.07<br>p = 0.010 | 1.03 ± 0.08 | NS | NS | ρ = 0.271<br>p = 0.027 |
| *ABCB4* | 1.41 ± 0.08<br>p = 0.024 | 1.45 ± 0.07 | NS | NS | NS | NS | NS | NS | NS | NS |
| *ABCB5* | 1.49 ± 0.16<br>p = 0.017 | 1.61 ± 0.23 | NS | NS | NS | NS | NS | NS | NS | NS |
| *ABCB6* | NS | NS | ρ = -0.257<br>p = 0.039 | NS | NS | NS | NS | 1.10 ± 0.04<br>p = 0.014 | 1.08 ± 0.05 | NS |
| *ABCB7* | NS | NS | ρ = -0.249<br>p = 0.045 | 1.07 ± 0.04<br>p = 0.045 | 1.09 ± 0.03 | 1.07 ± 0.04<br>p = 0.034 | 1.09 ± 0.03 | NS | NS | NS |
| *ABCC1*§ | NS | NS | NS | 1.08 ± 0.03<br>p = 0.029 | 1.06 ± 0.05 | 1.08 ± 0.04<br>p = 0.035 | 1.06 ± 0.05 | NS | NS | ρ = 0.245<br>p = 0.045 |
| *ABCC8*§ | NS | NS | NS | 1.35 ± 0.13<br>p < 0.001 | 1.57 ± 0.23 | 1.37 ± 0.16<br>p < 0.001 | 1.60 ± 0.21 | 1.40 ± 0.20<br>p = 0.004 | 1.55 ± 0.20 | ρ = -0.275<br>p = 0.024 |
| *ABCC10* | NS | NS | NS | NS | NS | 1.07 ± 0.04<br>p = 0.041 | 1.05 ± 0.03 | NS | NS | NS |
| *ABCC11*§ | NS | NS | NS | 1.19 ± 0.20<br>p = 0.042 | 1.31 ± 0.25 | 1.20 ± 0.20<br>p = 0.040 | 1.33 ± 0.27 | 1.20 ± 0.20<br>p = 0.006 | 1.36 ± 0.27 | ρ = -0.284<br>p = 0.020 |

*Only significant values are displayed. Values are mean ± standard deviation for the ratio of cycle threshold for a particular ATP-binding casette (ABC) gene to arithmetic mean of cycle threshold for all reference genes (ABC:REF). For statistical analyses of clinical characteristics and ABC transcript levels, a ABC:REF value was calculated for each sample. Therefore, the lower the ABC:REF ratio, the higher the ABC transcript level.*
*†p-values were calculated by a nonparametric Mann–Whitney U test. Mean ± standard deviation were calculated by analysis of variance to assess direction of associations.*
*‡p- and ρ-values by Spearman's correlation coefficient for significant associations are indicated.*
*§Genes used for validation study.*
*NS: Not significant.*

**Table 3. Significant associations of ATP-binding cassette transporter transcript levels with clinical characteristics of breast cancer patients in the evaluation set (cont.).**

| Gene | Lymph node metastasis† | | Tumor size‡ (mm) | Grade† | | ER† | | PR† | | Ki-67 expression‡ |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Absent* | *Present* | | *1 or 2* | *3* | *Positive* | *Negative* | *Positive* | *Negative* | |
| *ABCC13* | NS | NS | NS | NS | NS | 1.72 ± 0.23 | 1.93 ± 0.22 | 1.72 ± 0.24 | 1.94 ± 0.19 | NS |
| | | | | | | p = 0.002 | | p < 0.001 | | |
| *ABCG4* | 1.62 ± 0.08 | 1.67 ± 0.10 | NS | NS | NS | NS | NS | NS | NS | NS |
| | p = 0.045 | | | | | | | | | |

*Only significant values are displayed. Values are mean ± standard deviation for the ratio of cycle threshold for a particular ATP-binding casette (ABC) gene to arithmetic mean of cycle threshold for all reference genes (ABC:REF). For statistical analyses of clinical characteristics and ABC transcript levels, a ABC:REF value was calculated for each sample. Therefore, the lower the ABC:REF ratio, the higher the ABC transcript level.*
*†p-values were calculated by a nonparametric Mann–Whitney U test. Mean ± standard deviation were calculated by analysis of variance to assess direction of associations.*
*‡p- and p-values by Spearman's correlation coefficient for significant associations are indicated.*
*§Genes used for validation study.*
*NS: Not significant.*

molecular weight 576 kDa; The Human Protein Atlas [102]) and ABCC11 protein expression in breast tumors was already reported [24]. β-actin expression was used as an internal control for normalization of results. Analysis revealed high interindividual variability in expression of all examined ABC transporters (**Figure 2A–D**). However, protein expression of the four analyzed ABC transporters did not correlate with the corresponding transcript levels assessed by qPCR in the same tumor samples ($p > 0.05$; nonparametric Spearman's rank test).

## Discussion

Novel drugs, as well as identification of biomarkers of prognosis and chemotherapy efficacy, are a prerequisite for selection of the best treatment strategies to improve survival of cancer patients. Despite notable progress in research, MDR remains the main obstacle to successful cancer treatment. ABC transporters belong to one of the major factors leading to chemoresistance owing to their ability to efflux drugs outside of tumor cells diminishing the therapeutic effect [4]. Our study focused on identification of candidate biomarkers of prognosis and/or prediction of chemoresistance from the family of human ABC transporter genes. For this purpose, we have analyzed the gene-expression profile of all 49 human ABC transporters in tumors and adjacent nontumor tissues of post-treatment breast cancer patients and compared transcript levels with the established clinical prognostic and predictive factors. Selected candidate biomarkers were then validated in a pretreatment set of patients using two different methods (relative and absolute quantification).

The majority of ABC transporter genes have shown deregulation of expression in post-treatment tumors compared with nontumor control tissues. There are several studies on the assessment of transcript or protein levels of individual ABC transporters, mainly *ABCB1*, *ABCC1* and *ABCG2* in breast cancer [7,8,25]. However, a complete profile of gene expression of all ABC transporters in tissues from post-treatment breast cancer specimen was not previously reported. Recently, we published results of a study on gene-expression profiling of the complete ABC transporter family in colorectal cancer patients. Among others, *ABCA12* and *ABCC1* are upregulated in colorectal tumors compared with control tissues [20]. These two genes were upregulated in both pre- and post-treatment breast tumors compared with control tissues in the present study as well. The observed upregulation of *ABCC1* in breast tumors supports the previous report

**Table 4. Significant associations of ATP-binding casette transporters transcript levels with clinical characteristics of breast cancer patients in the validation set.**

| Gene | Tumor size† (mm) | Grade‡ | | ER† | | PR† | | HER2† | | p53 expression† | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *1 or 2* | *3* | *Positive* | *Negative* | *Positive* | *Negative* | *Positive* | *Negative* | *Positive* | *Negative* |
| *ABCA12* | NS | NS | NS | NS | NS | NS | NS | 1.16 ± 0.10<br>p = 0.016 | 1.21 ± 0.11 | NS | NS |
| *ABCC1* | ρ = -0.218<br>p = 0.031 | 1.09 ± 0.03§<br>p = 0.027§ | 1.07 ± 0.03§ | 1.09 ± 0.02§<br>p = 0.003§ | 1.07 ± 0.03§ | 1.09 ± 0.02<br>p = 0.010 | 1.07 ± 0.03 | NS | | 1.07 ± 0.03<br>p = 0.002 | 1.09 ± 0.03 |
| *ABCC8* | NS | 1.24 ± 0.11§<br>p = 0.038§ | 1.30 ± 0.12§ | 1.21 ± 0.08§<br>p < 0.001§ | 1.36 ± 0.11§ | 1.20 ± 0.08§<br>p < 0.001§ | 1.33 ± 0.11§ | 1.30 ± 0.13<br>p = 0.034 | 1.24 ± 0.11 | 1.31 ± 0.13<br>p = 0.009 | 1.23 ± 0.11 |
| *ABCC11* | NS | NS | NS | NS | NS | NS | NS | 1.17 ± 0.12<br>p = 0.019 | 1.10 ± 0.13 | NS | NS |
| *ABCD2* | ρ = -0.351<br>p < 0.001 | NS | NS | NS | NS | NS | NS | NS | | NS | NS |

*Only significant values are displayed. Values are mean ± standard deviation for the ratio of cycle threshold for a particular ATP-binding casette (ABC) gene to arithmetic mean of cycle threshold for all reference genes (ABC:REF). For statistical analyses of clinical characteristics and ABC transcript levels, a ABC:REF value was calculated for each sample. Therefore, the lower the ABC:REF ratio, the higher the ABC transcript level.*
*†ρ- and p-values by Spearman's correlation coefficient for significant associations are indicated.*
*‡p-values were calculated by nonparametric Mann–Whitney U test. Mean ± standard deviation were calculated by analysis of variance to assess direction of associations.*
*§Confirmed results from evaluation study.*
*NS: Not significant.*

showing a high percentage of ABCC1-positive breast tumors in both post-treatment (88%) and pretreatment (62%) specimens [12]. The downregulation of *ABCB1* observed in the post-treatment tumors by the present study confirms our previous results showing *ABCB1* downregulation in pretreatment specimens from another cohort of breast cancer patients [11]. We also confirmed the previously reported high *ABCC11* expression in breast tumors [26].

A number of associations between levels of ABC transporters in tumors and clinical data were observed in the evaluation set. Based on the number and strength of associations observed, we have chosen the six most interesting candidates (*ABCA12*, *ABCA13*, *ABCC1*, *ABCC8*, *ABCC11* and *ABCD2*) for a validation study in larger series of pretreatment patients. Significant associations of *ABCC1* and *ABCC8* with expression of hormonal receptors were confirmed in the pretreatment set. Associations with expression of hormonal receptors may suggest either prognostic and/or predictive importance of the above genes in breast cancer and hormonal control of their expression. It is a subject of our follow-up studies to test the mechanism of these associations. Patients with less aggressive tumors (grade 1 or 2) expressed significantly lower levels of *ABCC1* and higher levels of *ABCC8* than patients with more aggressive grade 3 tumors in both sets. Tumor grade is considered a validated prognostic factor in breast cancer [27] and thus *ABCC1*/*ABCC8* gene expression may cosegregate with the prognosis of breast cancer patients. Both genes also significantly associated with the Ki-67 protein expression in the evaluation set and p53 protein expression in the validation set. Ki-67 protein expression has predictive and prognostic value in NACT-treated patients and was suggested as a feasible marker for clinical practice [28]. A significant correlation between P-gp and Ki-67 protein staining has been previously reported [29]. However, other ABC transporters have not been studied in context with Ki-67 expression. Absence of association of ABCC1 (and ABCB1) protein expression with histological grade was observed before [30]. On the other hand, the previously suggested role of ABCC1 expression as a negative prognostic marker of both early-stage [12] and metastatic breast cancer [31] seems relevant to the association with high-grade and ER-negative tumors found by our study in both pre- and post-treatment patients. Association of high levels of *ABCC1* with factors of poor prognosis of breast cancer patients observed in our study is also in line with the recently proposed role of

ABCC1 in efflux of anticancer drugs [32,33] and with the reported association of ABCC1 protein level with shorter survival of breast cancer patients [34].

Unlike the majority of ABC transporters, *ABCC8* does not act as a drug transporter, but interacts with Kir6.2 to form an ATP-gated $K^+$ channel. Mutations in *ABCC8* have been associated with different forms of glycemic disorders, such as familial hyperinsulinemic hypoglycemia Type 1 and neonatal diabetes mellitus [20]. There are no data on the role of ABCC8 in cancer

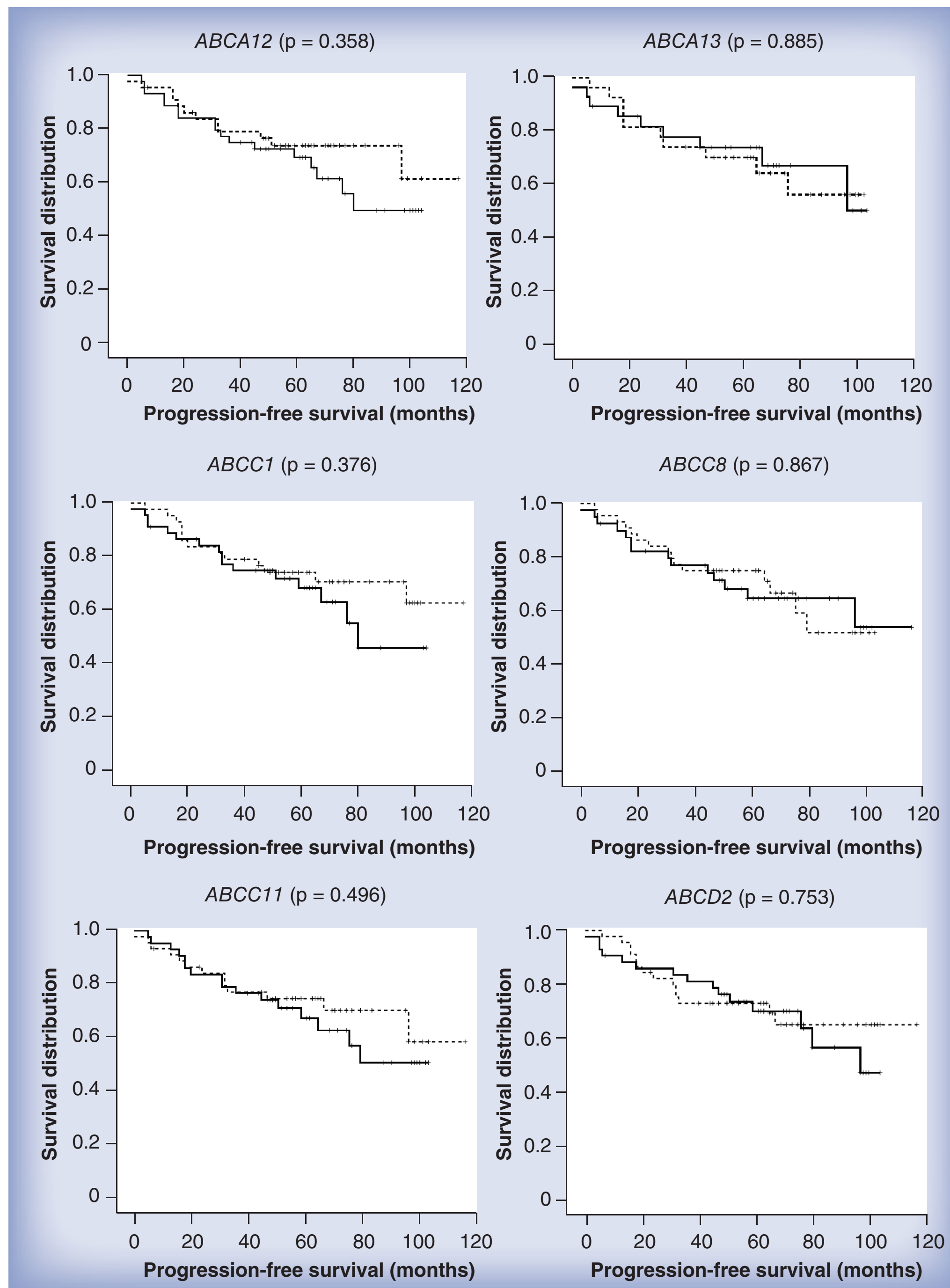


**Figure 1. Progression-free survival plots for ATP-binding cassette genes analyzed in the validation study.** Kaplan–Meier survival curves were plotted for patients (n = 88) divided into two groups according to the median of transcript levels in tumors. Dashed lines represent the group with higher transcript levels and solid lines represent the group with lower levels than median. Differences between groups were compared using log-rank test. The gene name and significant difference between groups are displayed.

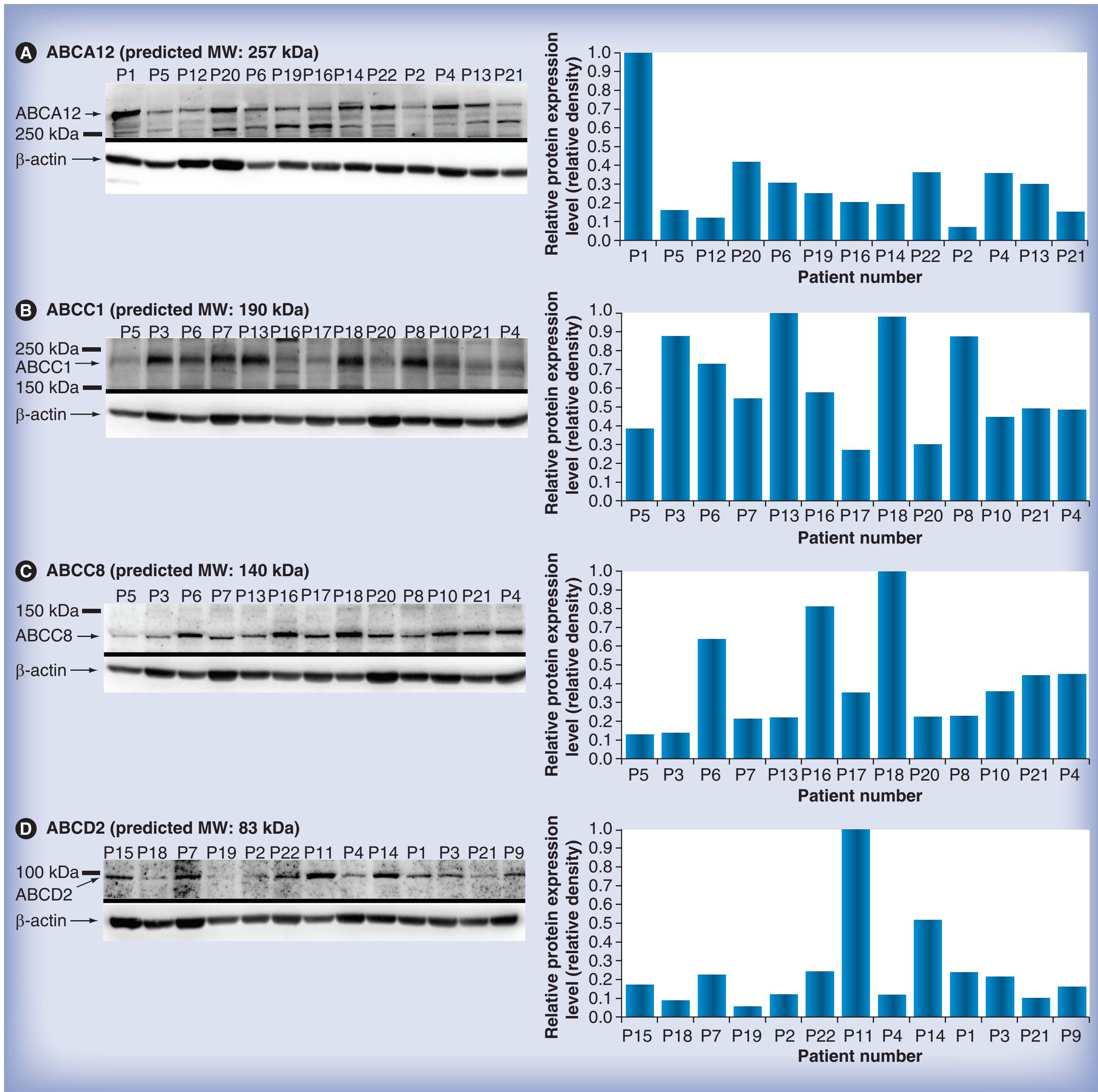


**Figure 2. Protein expression of ATP-binding cassette transporters in tumors.** Protein expression of **(A)** ABCA12, **(B)** ABCC1, **(C)** ABCC8 and **(D)** ABCD2 was assessed by immunoblotting (left) and evaluated by densitometry with normalization to β-actin (right) in representative set of breast tumors as described in the 'Patients & methods' section.
MW: Molecular weight; P: Patient.

progression. Moreover, the fact that low expression of *ABCC8* was associated with poor prognostic and predictive factors (ER-negative, grade 3 and Ki-67-high tumors) suggests a physiological rather than drug efflux-based mechanism. It should be the subject of future studies to identify the exact mechanism of the observed effects.

A number of associations between levels of ABC transporters and clinical data were observed in the evaluation set that were not confirmed by analysis of the candidates in the validation set and *vice versa*. Influence of drug pretreatment on tissue levels of ABC transporters, as well as by chance findings owing to multiple testing, cannot be excluded as a possible reason for these discrepancies.

Patients with stable or progressive disease after NACT had significantly higher levels of

*ABCA12* in control tissues than patients with partial or complete response in our study. A significantly higher expression of *ABCA12* (and *ABCA1*, *ABCC5*, *ABCC13*, *ABCB6* and *ABCC11*) in pretreatment tumors of breast cancer patients (n = 21) with residual disease compared with those who achieved complete pathological response to NACT was reported [14]. It is, however, unknown whether NACT can change the expression profile of *ABCA12*. Another candidate for a predictive marker suggested by our study, *ABCD2*, has not as yet been studied in breast cancer in such detail and has to be carefully validated. Quite recently, *ABCD3* has been suggested as a novel prostate cancer-associated gene that could, in part, be regulated by EGFR signaling [35]. None of the genes associated with response to NACT in the evaluation set (*ABCA12*, *ABCA13* and *ABCD2*) associated with PFS in the validation set.

Missing information about protein expression is a possible limitation of our study. According to the recent study, an immunohistochemical score consisting of semiquantitative measures of ER, PR, ERBB2 and Ki-67 provides a potentially useful prognostic tool that may further be extended to other genes [36]. The ABCC11 protein was already detected in breast tumors and was suggested as a possible predictive tool for chemotherapy choice [24]. We have observed a broad variability in protein expression of ABCA12, ABCC1, ABCC8 and ABCD2 between tumor samples from the training set. The lack of correlation between protein and transcript levels of candidate ABC transporters in the present study suggests that the gene-expression pattern shall be considered as an independent biomarker.

Different characteristics of evaluation versus validation sets may be seen as another limitation. On the other hand, by analysis of post- versus pre-treatment samples, we could validate the most common effects.

## Conclusion

Our study identified novel candidate markers of response to NACT (*ABCA12*, *ABCA13* and *ABCD2*) and revealed associations of *ABCC1* and *ABCC8* levels with established clinical markers of breast cancer prognosis, which have not been reported so far. Independent follow-up studies for identification of underlying mechanisms shall follow.

### Acknowledgements

*The authors would like to acknowledge generous support of J Kovář and the technical assistance of V Němcová-Furstová.*

### Financial & competing interests disclosure

*This work was supported by grants of the CSF No. P303/12/G163 and of the IGA No. NT/13679-4. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

*No writing assistance was utilized in the production of this manuscript.*

### Ethical conduct of research

*The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.*

### Executive summary

***Background***

- Active export of anticancer drugs from cancer cells driven mostly by ATP-binding cassette (ABC) transporters is one of the major mechanisms of drug resistance in human diseases.
- Understanding of the function of ABC transporters in tumors may improve the concept of individualized anticancer therapy.

***Patients & methods***

- Gene-expression profiles of all known human ABC transporters and their roles in breast cancer prognosis and therapy outcome in tissues from pre- and post-treatment patients were addressed by this study.

***Results***

- Six multidrug resistance-related genes (*ABCA2*, *ABCC1*, *ABCC4*, *ABCC5*, *ABCC10* and *ABCC11*) were significantly overexpressed in tumors compared with control tissues of post-treatment patients.
- *ABCC1* and *ABCC8* levels significantly associated with tumor grade and ER expression in both pre- and post-treatment patients.
- *ABCA12*, *ABCA13* and *ABCD2* levels significantly associated with response to neoadjuvant chemotherapy in post-treatment patients.
- Protein expression of ABCA12, ABCC8 and ABCD2 in tumor tissues of patients with breast carcinoma was observed for the first time.

***Conclusion***

- Biomarker candidates cosegregating with established clinical prognosis factors and with chemotherapy outcome of breast patients were revealed. Opportunities for their functional assessment and prospective pharmacogenetic profiling are now open.

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# The expression profile of ABC transporter genes and progression of breast carcinoma Supplementary material

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**Supplementary Table S1:** Therapeutic regimens used for treatment of the followed breast carcinoma patients

| **Therapy Regimen** | **Evaluation Set** | **Validation set** |
|---|---|---|
| *Neoadjuvant chemotherapy* | | |
| FAC/FEC[a] | 23 (33.8%) | None |
| FAC/FEC and taxane | 34 (50.0%) | |
| Other chemotherapy regimen[b] | 11 (16.2%) | |
| *Adjuvant Chemotherapy* | | |
| FAC/FEC[a] | | 29 (29.0%) |
| Other chemotherapy regimen[c] | | 19 (19.0%) |
| Hormonal therapy only | | 40 (40.0%) |
| Chemotherapy and hormonal therapy | | 29 (29.0%) |
| Untreated or data not available | | 10 (10.0%) |

Footnotes:

[a]FAC/FEC = combination of 5-fluorouracil, adriamycin/epirubicin and cyclophosphamide

[b]Other NACT regimens: adriamycin and taxane (AT, n=7), paclitaxel, estramustine and carboplatin (TEC, n=1) regimen and hormonal treatment only (n=3)

[c]Other adjuvant regimens: cyclophosphamide, methotrexate and 5-fluorouracil (CMF, n=6), FAC/FEC and taxane (n=10), adriamycin and taxane (n=2) and taxane only (n=1). Three patients received Herceptin in the course of adjuvant therapy.

**Supplementary Table 2S:** List of gene expression assays used for this study (reference 20)

| Gene Symbol | OMIM number | Assay ID | RefSeq | Exon Boundary | Amplicon Length (bp) |
|---|---|---|---|---|---|
| ***EIF2B1*** | 606686 | Hs00426752_m1 | NM_001414.3 | 4 – 5 | 75 |
| ***IPO8*** | 605600 | Hs00183533_m1 | NM_006390.3 | 20 – 21 | 71 |
| ***MRPL19*** | 611832 | Hs00608519_m1 | NM_014763.3 | 2 – 3 | 72 |
| ***UBB*** | 191339 | Hs00430290_m1 | NM_018955.2 | 1 – 2 | 120 |
| ***ABCA1*** | 600046 | Hs00194045_m1 | NM_005502.3 | 30 – 31 | 125 |
| ***ABCA2*** | 600047 | Hs00242232_m1 | NM_001606.4 | 10 – 11 | 58 |
| ***ABCA3*** | 601615 | Hs00184543_m1 | NM_001089.2 | 19 – 20 | 77 |
| ***ABCA4*** | 601691 | Hs00184367_m1 | NM_000350.2 | 38 – 39 | 71 |
| ***ABCA5*** | 612503 | Hs00363322_m1 | NM_172232.2 | 18 – 19 | 100 |
| ***ABCA6*** | 612504 | Hs00365329_m1 | NM_080284.2 | 26 – 27 | 83 |
| ***ABCA7*** | 605414 | Hs00185303_m1 | NM_019112.3 | 40 – 41 | 80 |
| ***ABCA8*** | 612505 | Hs00992371_m1 | NM_007168.2 | 28 – 29 | 85 |
| ***ABCA9*** | 612507 | Hs00329320_m1 | NM_080283.3 | 2 – 3 | 145 |
| ***ABCA10*** | 612508 | Hs00365268_m1 | NM_080282.3 | 3 – 4 | 127 |
| ***ABCA12*** | 607800 | Hs00292421_m1 | NM_015657.3 | 1 – 2 | 77 |
| ***ABCA13*** | 607807 | Hs01110169_m1 | NM_152701.3 | 32 – 33 | 80 |
| ***ABCB1*** | 171050 | Hs00184491_m1 | M14758.1 | 23 – 24 | 110 |
| ***ABCB2*** | 170260 | Hs00388677_m1 | NM_000593.5 | 5 – 6 | 60 |
| ***ABCB3*** | 170261 | Hs00241060_m1 | NM_000544.3 | 5 – 6 | 66 |
| ***ABCB4*** | 171060 | Hs00240956_m1 | NM_000443.3 | 2 – 3 | 73 |
| ***ABCB5*** | 611785 | Hs00698751_m1 | NM_178559.5 | 11 – 12 | 90 |
| ***ABCB6*** | 605452 | Hs00180568_m1 | NM_005689.2 | 14 – 15 | 60 |
| ***ABCB7*** | 300135 | Hs00188776_m1 | NM_004299.3 | 13 – 14 | 92 |
| ***ABCB8*** | 605464 | Hs00185159_m1 | NM_007188.3 | 14 – 15 | 74 |
| ***ABCB9*** | 605453 | Hs00608640_m1 | NM_203444.2 | 9 – 10 | 75 |
| ***ABCB10*** | 605454 | Hs00429240_m1 | NM_012089.2 | 2 – 3 | 133 |
| ***ABCB11*** | 603201 | Hs00184824_m1 | NM_003742.2 | 21 – 22 | 63 |
| ***ABCC1*** | 158343 | Hs00219905_m1 | NM_004996.3 | 24 – 25 | 74 |
| ***ABCC2*** | 601107 | Hs00166123_m1 | NM_000392.3 | 25 – 26 | 75 |
| ***ABCC3*** | 604323 | Hs00358656_m1 | NM_001144070.1 | 8 – 9 | 98 |
| ***ABCC4*** | 605250 | Hs00195260_m1 | NM_005845.3 | 25 – 26 | 86 |
| ***ABCC5*** | 605251 | Hs00981089_m1 | NM_001023587.1 | 4 – 5 | 68 |
| ***ABCC6*** | 603234 | Hs00184566_m1 | NM_001171.5 | 17 – 18 | 56 |
| ***ABCC7*** | 602421 | Hs00357011_m1 | NM_000492.3 | 21 – 22 | 93 |
| ***ABCC8*** | 600509 | Hs00165861_m1 | NM_000352.3 | 16 – 17 | 137 |
| ***ABCC9*** | 601439 | Hs00245832_m1 | NM_005691.2 | 28 – 29 | 70 |
| ***ABCC10*** | 612509 | Hs00375716_m1 | NM_033450.2 | 16 – 17 | 142 |
| ***ABCC11*** | 607040 | Hs01090768_m1 | NM_032583.3 | 26 – 27 | 76 |
| ***ABCC12*** | 607041 | Hs00264354_m1 | NM_033226.2 | 7 – 8 | 90 |
| ***ABCC13*** | 608835 | Hs01051917_m1 | NR_003087.1 | 1 – 2 | 130 |
| ***ABCD1*** | 300371 | Hs00163610_m1 | NM_000033.3 | 1 – 2 | 101 |
| ***ABCD2*** | 601081 | Hs00193054_m1 | NM_005164.3 | 2 – 3 | 109 |
| ***ABCD3*** | 170995 | Hs00161065_m1 | NM_002858.3 | 20 – 21 | 91 |
| ***ABCD4*** | 603214 | Hs00245340_m1 | NM_005050.3 | 11 – 12 | 117 |

| | | | | | |
|---|---|---|---|---|---|
| ***ABCE1*** | 601213 | Hs01009190_m1 | NM_001040876.1 | 1 – 2 | 91 |
| ***ABCF1*** | 603429 | Hs00153703_m1 | NM_001025091.1 | 20 – 21 | 69 |
| ***ABCF2*** | 612510 | Hs00606493_m1 | NM_005692.3 | 14 – 15 | 113 |
| ***ABCF3*** | 55324* | Hs00217977_m1 | NM_018358.2 | 16 – 17 | 61 |
| ***ABCG1*** | 603076 | Hs00245154_m1 | NM_004915.3 | 5 – 6 | 58 |
| ***ABCG2*** | 603756 | Hs00184979_m1 | NM_004827.2 | 5 – 6 | 92 |
| ***ABCG4*** | 607784 | Hs00223446_m1 | NM_001142505.1 | 9 – 10 | 93 |
| ***ABCG5*** | 605459 | Hs00223686_m1 | NM_022436.2 | 10 – 11 | 60 |
| ***ABCG8*** | 605460 | Hs00223690_m1 | NM_022437.2 | 6 – 7 | 63 |

Footnotes:

*MIM number not available, Gene ID used instead

# Příloha č. 6:

Brynychova V, **Hlavac V**, Ehrlichova M, Vaclavikova R, Nemcova-Furstova V, Pecha V, Trnkova M, Mrhalova M, Kodet R, Vrana D, Gatek J, Bendova M, Vernerova Z, Kovar J, Soucek P

**Transcript expression and genetic variability analysis of caspases in breast carcinomas suggests CASP9 as the most interesting target**

Clin Chem Lab Med. 2017 Jan 1;55(1):111-122 [IF: 3,017] 5 %*)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

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# Transcript expression and genetic variability analysis of caspases in breast carcinomas suggests CASP9 as the most interesting target

DOI 10.1515/cclm-2016-0271
Received April 5, 2016; accepted May 17, 2016; previously published online June 21, 2016

**Abstract**

**Background:** Apoptosis plays a critical role in cancer cell survival and tumor development. We provide a hypothesis-generating screen for further research by exploring the expression profile and genetic variability of caspases (2, 3, 7, 8, 9, and 10) in breast carcinoma patients. This study addressed isoform-specific caspase transcript expression and genetic variability in regulatory sequences of caspases 2 and 9.

**Methods:** Gene expression profiling was performed by quantitative real-time PCR in tumor and paired non-malignant tissues of two independent groups of patients. Genetic variability was determined by high resolution melting, allelic discrimination, and sequencing analysis in tumor and peripheral blood lymphocyte DNA of the patients.

**Results:** CASP3 A+B and S isoforms were over-expressed in tumors of both patient groups. The CASP9 transcript was down-regulated in tumors of both groups of patients and significantly associated with expression of hormonal receptors and with the presence of rs4645978-rs2020903-rs4646034 haplotype in the *CASP9* gene. Patients with a low intratumoral CASP9A/B isoform expression ratio (predicted to shift equilibrium towards anti-apoptotic isoform) subsequently treated with adjuvant chemotherapy had a significantly shorter disease-free survival than those with the high ratio (p=0.04). Inheritance of CC genotype of rs2020903 in *CASP9* was associated with progesterone receptor expression in tumors (p=0.003).

**Conclusions:** Genetic variability in *CASP9* and expression of its splicing variants present targets for further study.

**Keywords:** alternative splicing; breast carcinoma; caspases; prognosis; transcript.

## Introduction

Breast carcinoma is the most common cancer in women (OMIM:114480 [1]). Despite considerable progress in pharmacogenomics, valid biomarkers for prediction of the response of patients to drugs used to treat breast carcinoma (e.g. taxanes, paclitaxel, and docetaxel) are missing [2].

Deregulation of apoptosis may contribute to carcinogenesis and chemoresistance [3, 4]. Caspases are cysteine proteases critical for apoptosis. Caspase-3 (coded by *CASP3* gene, OMIM:600636) executes both extrinsic (death receptor-induced) and intrinsic (mitochondrial) apoptotic

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pathways. Caspase-7 (*CASP7*, OMIM:601761) is another important executive caspase [5]. Caspases-9 (*CASP9*, OMIM:602234), -8 (*CASP8*, OMIM:601763), and -10 (*CASP10*, OMIM:601762) are initiator caspases that activate caspase-3 and caspase-7. Caspase-9 is activated in the mitochondrial pathway, and caspases-8 and -10 interact with tumor necrosis factor family proteins and drive extrinsic apoptosis [3]. Caspase-2 (*CASP2*, OMIM:600639) is activated in both apoptotic pathways, and its role in cell cycle regulation and tumor suppression has been widely discussed [6]. Altered caspase expression is associated with the resistance of breast carcinoma cells towards taxanes in vitro [7–9]. From this point of view, the study of caspase expression in breast carcinoma raises a considerable interest.

We have recently found a lack of association of expression of (pro-apoptotic) isoform of caspase-2, CASP2L, and its alternative (anti-apoptotic) isoform CASP2S with the response to neoadjuvant chemotherapy in breast carcinoma patients [10]. Alternative caspase variants with pro-survival function have also been characterized in caspase-3, caspase-8, and caspase-9 [11–15]. However, the relevance of these isoforms for breast carcinoma biology and their importance for cancer therapy is underexplored.

Although genetic alterations in caspase genes are commonly found in carcinomas including breast cancer [16, 17], their effect on caspase transcription and mainly alternative splicing in breast carcinoma is virtually unknown.

This study explored transcript levels of caspases-2, -3, -7, -8, -9, and -10 in tumors and paired adjacent non-malignant tissues of breast carcinoma patients. The expressions of transcript variants CASP3A, CASP3B, CASP3S, CASP8L, CASP9A, and CASP9B were also determined. Transcript levels were compared with clinical data and patients' response to the therapy. Association of CASP9A/B isoform expression ratio with survival of patients was observed. Therefore, genetic alterations in regulatory regions of caspase 9 were assessed to gain functional insight into their importance for expression and alternative splicing in breast carcinoma samples. Due to the over-expression of CASP2L in tumors of breast carcinoma patients demonstrated in our previous work [10], we also analyzed regulatory regions of the *CASP2* gene.

# Materials and methods

## Patients

The study included 128 mammary carcinoma tissue samples and 65 paired adjacent non-malignant tissues without morphological signs of carcinoma. Samples were collected and snap frozen during primary surgery in The Faculty Hospital Motol and Institute for the Care for Mother and Child (Prague, Czech Republic) in the period between 2003 and 2009. Blood samples from 615 patients for the genotyping validation study were collected from patients treated in The Faculty Hospital Motol, Institute for the Care for Mother and Child, The Faculty Hospital Kralovske Vinohrady (Prague, Czech Republic), and The Hospital Atlas (Zlin, Czech Republic). The processing of the tissue and blood samples was described in detail previously [10, 18]. Histological classification of carcinomas was performed according to standard diagnostic procedures [19]. Expression of estrogen and progesterone receptors was assessed immunohistochemically with the 1% cut-off value for classification of tumors as hormone receptor positive. ERBB2 (OMIM:164870) status was defined as positive in samples with immunohistochemical score 2+ or 3+ confirmed by fluorescence in situ hybridization (FISH) or silver in situ hybridization (SISH) analysis.

The patients were divided into three subtypes according to the hormone receptor expression and ERBB2 status: triple-negative (TNBC) subtype (associated with the worst prognosis), ERBB2 positive subtype, and luminal subtype [20].

Samples from 88 patients were collected during the primary surgery without any prior chemotherapy or hormonal therapy pretreatment (PS group). Samples from the second group of patients (n=40) were collected during the primary surgery after chemotherapy by neoadjuvant cytotoxic therapy (NACT) regimen containing taxanes or taxanes in combination with 5-fluorouracil, anthracycline, and cyclophosphamide (NACT group). Disease-free survival (DFS) was defined as the time elapsed between surgery and disease recurrence. Response to the neoadjuvant therapy in the NACT group was evaluated based on ultrasonography performed before and after the therapy.

All patients were asked to read and sign an informed consent and the study was approved by the Ethical Commission of the National Institute of Public Health in Prague.

## DNA analysis of splice and regulatory sites

Genomic DNA was isolated from mammary carcinoma tissues with the help of AllPrep® DNA/RNA/Protein Mini Kit (Qiagen, Hildesheim, Germany) according to the manufacturer's protocol and from corresponding peripheral blood lymphocytes by the phenol/chloroform extraction method [21]. DNA was quantified by Quant-iT PicoGreen DNA Assay Kit (Invitrogen).

Splicing and regulatory regions analyzed in *CASP2* and *CASP9* were selected according to the previously published studies [15, 22, 23] and are summarized in Figure 1. Together, nineteen single nucleotide polymorphisms (SNPs) located in these regions with minor allele frequency (MAF) ≥1% were included in the analysis (Supplementary Table S1). Promoter, splicing, and regulatory regions (In100) in *CASP2* were analyzed by direct sequencing as well as the SNPs in *CASP9* promoter and splicing elements, enhancers, and silencers in *CASP9* gene (Figure 1). SNPs rs4661636 and rs6685648 were determined by high resolution melting analysis (HRM). TaqMan SNP genotyping assay C___2845923_10 was used to determine rs2020903 in the validation study (see below). Primer sequences and PCR conditions for HRM and sequencing analyses are available upon request. PCR conditions and primers for amplification of region of alternative exon 9 and In100 region in *CASP2* were published recently [10].

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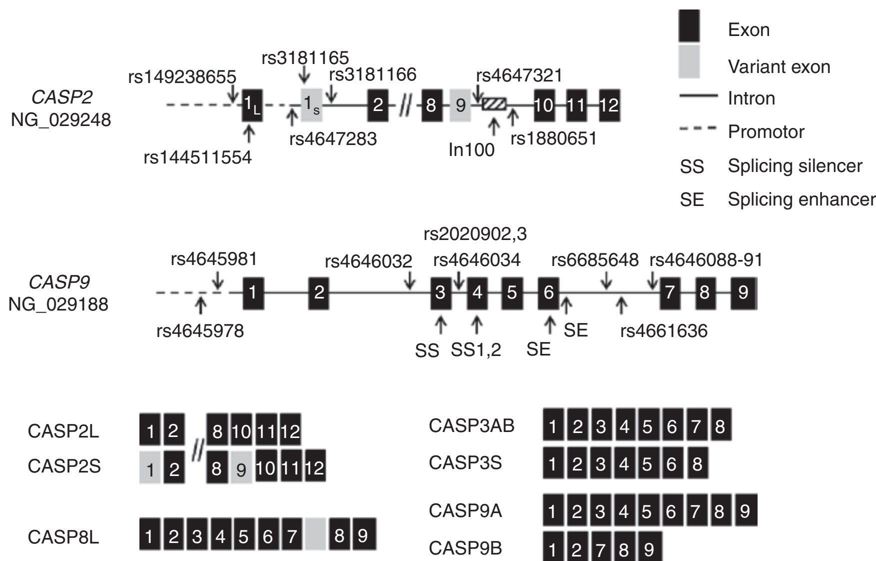


**Figure 1:** Genetic alterations and splice variants analyzed by the study.

PCR products for analysis by sequencing were checked on 1% agarose gel electrophoresis and then amplified using BigDye Terminator v3.1 Cycle Sequencing Kit according to producer's protocol (Life Technologies). Sequencing was performed using ABI3100 Genetic Analyzer and evaluated by Sequencing Analysis Software v5.2 (Life Technologies). The Type It HRM kit (Qiagen) and RotorGene 6000 (Corbett Reseach, Sydney, Australia) were used for HRM analysis and Viia7 Real-Time PCR System with 384-well block (Life Technologies) for TaqMan genotyping. Ten percent of randomly selected samples were reanalyzed with 100% concordance of results. SNPs were first analyzed in a small-scale exploratory study (n=60) and successful hits were confirmed in the large-scale validation study (n=615).

## Isolation of total RNA and cDNA preparation

Total RNA was isolated from macrodissected fresh frozen tissues using Trizol Reagent (Invitrogen, CA, USA), quantified by Quant-iT RiboGreen RNA Assay Kit (Invitrogen) using Infinite M200 multiplate reader (Tecan Group Ltd, Männedorf, Switzerland) and RNA integrity was checked by Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Assay Kit (Agilent Technologies, Inc., CA, USA). Total RNA was transcribed to cDNA with random hexamer primers as described previously [10]. Due to the limited amount of tissue samples, a multiplex PCR preamplification of post-treatment cDNA samples was performed using TaqMan PreAmp Master Mix Kit following manufacturer's instructions (Life Technologies) and preamplification uniformity was assessed as described previously [24].

## Quantitative real-time PCR

Real-time PCR quantification (qPCR) of mammary carcinomas and paired adjacent non-malignant samples was carried out in ViiA7 Real-Time PCR System with 384-well block (Life Technologies) as described before [10]. For qPCR of caspase transcripts and reference genes *EIF2B1*, *MRPL19*, and *IPO8* [24], commercially available or newly designed TaqMan Gene Expression Assays were used (Supplementary Table S2). Specific DNA fragments (standards) of CASP2L, CASP2S, CASP3A and 3B, CASP3S, CASP8L, CASP9A, and CASP9B transcript variants were prepared from cDNA by PCR, purified from the gel according their size, and confirmed by sequencing. Specificity of isoforms-specific assays was tested by qPCR reactions containing serially diluted standards and by direct sequencing of qPCR products. Efficiencies of all assays were between 90% and 100%. Samples were amplified in duplicates and reanalyzed if the variation between duplicates was larger than 0.5 quantitation cycle (Cq). The non-template control contained water instead of cDNA. Negative cDNA synthesis controls (RNA transcribed without reverse transcriptase) were also employed to reveal possible carry-over contamination. The qPCR study design adhered to the MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments [25]).

## Statistical analysis

Differences in gene expression between tumors and non-malignant tissues were analyzed by REST 2009 Software (Qiagen) considering both normalization to selected reference genes and reaction efficiencies of all gene expression assays. Associations of transcript levels with clinical data were analyzed by non-parametric tests (Kruskal-Wallis, Mann-Whitney, and Spearman rank). Ratios of Cq values of genes of interest and mean value of three reference genes were calculated for this purpose. Tested variables are presented in Table 1.

For DFS analysis, transcript levels in mammary tumors were divided into groups according to median. The Kaplan-Meier plot and the log-rank test were used to compare transcript levels and ratios of splicing variants with DFS of unselected patients or patients divided by the therapy type. Analogous methods were used for DFS analysis in the validation study of genetic variability.

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**Table 1:** Clinical characteristics of the studied breast carcinoma patients used for gene expression profiling.

| Characteristics | PS group[a] | NACT group[a] |
|---|---|---|
| Mean age at diagnosis, years±SD[b] | 62.2±10.5 | 52.8±8.3 |
| Menopausal status | | |
| Premenopausal | 8 (9.1) | 18 (45.0) |
| Postmenopausal | 80 (90.9) | 22 (55.0) |
| Histological type | | |
| Invasive ductal carcinoma | 76 (86.4) | 28 (70.0) |
| Other type[c] | 12 (13.6) | 12 (30.0) |
| Stage | | |
| I | 34 (38.6) | 7 (17.5) |
| II | 39 (44.3) | 21 (52.5) |
| III | 11 (12.5) | 10 (25.0) |
| IV | 0 | 1 (2.5) |
| Not assessed | 4 (4.6) | 1 (2.5) |
| Average tumor size, mm±SD[b] | 18.79.2 | 30.1±16.3 |
| Lymph node metastasis | | |
| Positive (pN1–3) | 33 (37.5) | 26 (65.0) |
| Negative (pN0) | 51 (58.0) | 14 (35.0) |
| pNx | 4 (4.5) | 0 |
| Histological grade | | |
| 1 | 15 (17.1) | 2 (5.0) |
| 2 | 53 (60.2) | 25 (62.5) |
| 3 | 17 (19.3) | 13 (32.5) |
| Not assessed | 3 (3.4) | 0 |
| Estrogen receptor expression | | |
| Positive | 60 (68.2) | 28 (70.0) |
| Negative | 28 (31.8) | 12 (30.0) |
| Progesterone receptor expression | | |
| Positive | 52 (59.1) | 29 (72.5) |
| Negative | 36 (40.9) | 11 (27.5) |
| ERBB2 status | | |
| Positive | 25 (28.4) | 11 (27.5) |
| Negative | 63 (71.6) | 29 (72.5) |
| Subtype | | |
| Luminal | 62 (70.5) | 31 (77.5) |
| ERBB2 | 11 (12.5) | 5 (12.5) |
| Triple negative | 15 (17.0) | 4 (10.0) |
| Response to neoadjuvant chemotherapy | Not applicable | |
| Complete or partial response | | 14 (35.0) |
| Stable disease or progression | | 17 (42.5) |
| Not available | | 9 (22.5) |

[a]Number of patients with percentage in parentheses. [b]SD, standard deviation. [c]Other tumor types involved invasive lobular (6/8), mucinous (2/1), ductal in situ (2/0), medullary (1/0), papillary (1/0), metaplastic (0/1) and solid neuroendocrine (0/2) carcinomas.

The Mann-Whitney test was used for analysis of associations between caspase isoform transcript levels and SNPs in *CASP2* and *CASP9*. The Kruskal-Wallis and the Pearson $\chi^2$-tests in the exploratory SNP study and the Mantel-Haenszel common odds ratio estimate (OR) with 95% confidence intervals (95% CI) in the validation SNP study were used for analysis of associations of SNPs with clinical data. Co-dominant, dominant, and recessive genetic models were evaluated in the validation SNP study. Tested variables are presented in Supplementary Table S3. The functional relevance of the clinically relevant SNPs was analyzed in silico by HaploReg v4 [26].

All p-values were obtained from two-sided tests. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS v16.0 software (SPSS Inc., Chicago, IL, USA [27]). The correction for false discovery rate (FDR) according to Benjamini and Hochberg [28] was applied to all analyses except the DFS analysis.

# Results

## Study population for tissue study

The tissue study population consisted of 128 patients with histologically confirmed breast carcinoma (clinical data listed in Table 1). The NACT group of patients had significantly younger age at diagnosis, prevalence of premenopausal status, larger and poorly differentiated (high grade) tumors and higher number of patients with lymph nodes affected by metastasis in comparison with the PS group.

The mean follow-up of the PS group of patients was 58.8 months. DFS was not analyzed in the NACT group of patients because of the limited number of patients in this group with complete follow-up data.

## Differences in gene expression of caspases between tumor and control tissues

Transcript levels of CASP2, CASP3 (including their isoforms), and CASP7 were significantly higher in tumors compared to paired non-malignant tissues in the PS group (Figure 2). In the opposite, CASP9 and CASP10 levels were significantly lower in tumors of this group of patients. The rest of evaluated caspase levels did not differ (p>0.05).

Over-expression of CASP3 isoforms (A+B and S) and total CASP9 down-regulation in tumors were also found in the NACT group of patients. Other followed caspases were not deregulated in tumor tissues (p>0.05).

## Associations of transcript levels of caspases with clinical characteristics of patients

Intratumoral levels of caspase transcripts were correlated with available clinical data of patients. All clinical data have been analyzed in both groups of patients, but to retain concise style only significant values are displayed in Table 2.

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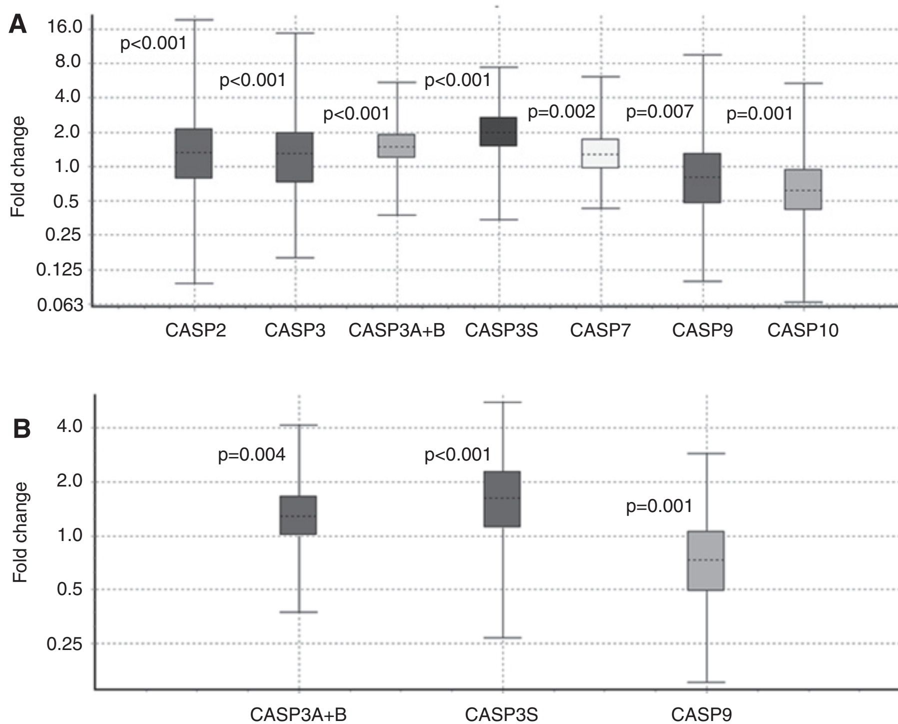


**Figure 2:** Significant differences (fold changes) in the relative transcript levels between paired samples of tumor and control tissues from breast carcinoma patients.
(A) PS group ($p<0.001$, CASP2; $p<0.001$, CASP3; $p<0.001$, CASP3A+B; $p<0.001$, CASP3S; $p=0.002$, CASP7; $p=0.007$, CASP9; $p<0.001$, CASP10). (B) NACT group ($p=0.004$, CASP3A+B; $p<0.001$, CASP3S; $p=0.001$, CASP9). The fold change more than 1 represents over-expression and the fold change less than to 1 represents down-regulation. The box area encompasses 50% of all observations, the dotted line represents the sample median and the error bars indicate maximum and minimum values.

Higher levels of CASP2, CASP3A+B, and CASP9B but lower levels of CASP7, CASP9, and CASP9A were found in tumors without expression of hormonal receptors when compared to tumors expressing hormonal receptors (Table 2A). Association of CASP9 level with expression of hormonal receptors was also observed in the NACT group of patients, although it did not pass the FDR test for multiple testing (Table 2B). The rest of significant associations were observed in only one of the evaluated sets of patients (Table 2A,B).

Transcript levels of caspases in tumor tissues did not significantly modify the DFS of patients in the PS group (n=77, Supplementary Figure S1A-M). However, a subgroup of patients with lower CASP9A/B ratio than the median (i.e. shift towards anti-apoptotic isoform) subsequently treated with adjuvant chemotherapy had significantly shorter DFS than patients with a higher ratio (Figure 3). Transcript levels of caspases did not significantly modify the response to the neoadjuvant treatment in the NACT group (CASP2, $p=0.888$; CASP3, $p=0.905$; CASP3AB, $p=0.131$; CASP3S, $p=0.190$; CASP3AB/S ratio, $p=0.968$; CASP7, $p=0.204$; CASP8, $p=0.341$; CASP8L, $p=0.341$; CASP9, $p=0.634$; CASP9A, $p=0.874$; CASP9B, $p=0.250$; CASP9A/B ratio, $p=1.000$; CASP10, $p=0.112$).

## Analysis of genetic variability in CASP2 and CASP9

Genomic DNA samples from tumor tissues and peripheral blood lymphocytes of 60 breast carcinoma patients from the PS group were used for SNP analysis of *CASP2* and *CASP9* genes in the exploratory SNP study. All regions and SNPs analyzed in *CASP2* and *CASP9* (summarized in Figure 1 and Supplementary Table S1) were selected using the following criteria: (1) localization in promoters, enhancers, silencers or splicing elements (according to UCSC Genome Browser, https://genome.ucsc.edu/), (2) previously published data (when available) [15, 22, 23], (3) minor allele frequency in Caucasians higher or equal to 1% (Supplementary Table S1). Sequence data of the alternative exon 9 and In100 region of *CASP2* found in peripheral blood lymphocytes [10] were used to supplement the data from tumor samples obtained by this study.

No differences in DNA alterations between samples from tumor tissues and peripheral blood lymphocytes were observed in any of the genes (Supplementary Table S4). Four new SNPs were found and characterized in *CASP2* promoters (Table 3).

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**Table 2:** Associations of transcript levels of caspases in tumors with clinical data of breast carcinoma patients.

| Gene | Grade | | ERBB-2 status | | ER expression | | PR expression | | Subtype | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 or 2 n=68 | 3 n=17 | Positive n=25 | Negative n=63 | Positive n=60 | Negative n=28 | Positive n=52 | Negative n=36 | Luminal n=62 | ERBB-2 n=11 | TNBC n=15R |
| (A) PS group | | | | | | | | | | | |
| *CASP2* | NS | | NS | | 1.01±0.02 p=0.020[a] | 0.99±0.02 | 1.01±0.02 p=0.008[a] | 0.99±0.02 | 1.01±0.02 p=0.033 | 1.00±0.03 | 0.99±0.01 |
| *CASP3A+B* | NS | | NS | | 1.02±0.02 p=0.002[a] | 1.01±0.02 | 1.02±0.01 p=0.002[a] | 1.01±0.02 | 1.02±0.02 p=0.001[a] | 1.00±0.01 | 1.01±0.02 |
| *CASP7* | NS | | NS | | 1.05±0.02 p=0.002[a] | 1.07±0.02 | 1.05±0.01 p=0.008[a] | 1.07±0.02 | 1.05±0.02 p=0.008[a] | 1.06±0.02 | 1.07±0.02 |
| *CASP9* | NS | | 1.06±0.02 p=0.011[a] | 1.05±0.02 | 1.05±0.02 p<0.001[a] | 1.07±0.02 | 1.05±0.02 p=0.002[a] | 1.06±0.02 | 1.05±0.02 p=0.001[a] | 1.07±0.02 | 1.07±0.02 |
| *CASP9A/B* | 0.92±0.02 p=0.011[a] | 0.94±0.02 | NS | | 0.92±0.02 p<0.001[a] | 0.94±0.02 | 0.91±0.02 p<0.001[a] | 0.93±0.02 | 0.91±0.02 p<0.001[a] | 0.94±0.02 | 0.94±0.02 |

| Gene | ER expression | | PR expression | | ERBB-2 status | |
|---|---|---|---|---|---|---|
| | Positive n=28 | Negative n=12 | Positive n=29 | Negative n=11 | Positive n=11 | Negative n=29 |
| (B) NACT group | | | | | | |
| *CASP8* | NS | | NS | | 1.40±0.04 p=0.035 | 1.37±0.04 |
| *CASP9* | 1.03±0.03 p=0.031 | 1.05±0.02 | 1.03±0.03 p=0.035 | 1.05±0.02 | 1.03±0.02 p=0.035 | 1.05±0.02 |
| *CASP9A/B* | NS | | NS | | 0.93±0.02 p=0.025 | 0.92±0.01 |

All clinical data have been analyzed in both groups of patients, but to retain concise style only significant values are displayed. Values of caspase expression are mean±standard deviation of the ratio of cycle threshold for a target gene to the arithmetic mean of cycle threshold of all reference genes. Therefore, the lower is the ratio the higher is the respective transcript level. Cycle threshold values were used directly to calculate CASP9 variants ratios, therefore higher value means higher expression of anti-apoptotic variant than pro-apoptotic variant. n, number of patients in the group; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple negative breast carcinoma. p-Values were calculated by the Kruskal-Wallis test. [a]These results passed the Benjamini-Hochberg FDR test for multiple testing (q=0.032). Significant results observed in both patient groups are highlighted in gray.

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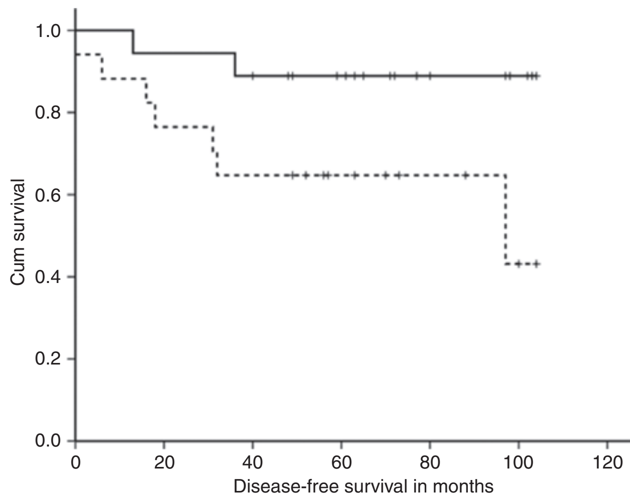


**Figure 3:** Association between CASP9A/B ratio in tumors and disease-free survival of breast carcinoma patients.
Kaplan-Meier survival curves for patients with the intratumoral CASP9A/B ratio below or equal the median (solid line, n=18) vs. patients with the ratio above the median (dashed line, n=17) are displayed. The difference in the mean DFS between the compared groups of patients was significant (p=0.04, log-rank test).

Tumors from carriers of the GG genotype in rs4645978, the CC genotype in rs4646032, the CC genotype in rs2020903, and the AA genotype in rs4646034 had significantly lower *CASP9* transcript levels than tumors from carriers of the alternative alleles. Conversely, tumors from carriers of the GG genotype in rs4661636 had significantly higher *CASP9* transcript levels than tumors from carriers of the A allele (Table 4).

All clinical data have been analyzed against the followed SNPs, but to retain concise style only significant values are displayed in Tables 5, S5, and S6. From the comparison of SNPs with clinical data in the exploratory study, the most notable association was that of rs4645978-rs2020903-rs4646034 SNPs in *CASP9* with ER/PR expression (Supplementary Table S5). This association seemed to reflect the previously established links between the CASP9 transcript and the expression of estrogen and progesterone receptors (Table 2) and between the CASP9 transcript and these SNPs (Table 4). The significance of the *CASP9* rs4645978-rs2020903-rs4646034 haplotype was therefore further validated in peripheral blood lymphocyte DNA samples obtained from 615 breast carcinoma patients (clinical data listed in Supplementary Table S3).

Inheritance of rs2020903 SNP in *CASP9* was significantly associated with the progesterone receptor and ERBB2 expression (but not with estrogen receptor expression, Supplementary Table S6), presence of lymph node metastasis, and TNBC subtype of breast carcinoma (Table 5). The association of rs2020903 with the progesterone receptor expression was observed in all genetic models tested, and a tendency towards a decrease in the risk of having progesterone receptor negative tumor with a number of inherited alleles was obvious (p=0.050 for one C allele vs. p=0.003 for both alleles, Table 5). The remaining associations reached rather marginal significances that did not pass the FDR test for multiple testing.

## Discussion

Bearing in mind a critical role of apoptosis in cancer cell survival and tumor development [4], we pursued the hypothesis-generating screen to provide candidates for further research of the prognostic and predictive role of caspases (i.e. the main executors of apoptosis) in breast carcinoma.

**Table 3:** Novel *CASP2* SNPs found in breast carcinoma patients.

| SNPs | Location, type[a] | Genotype | n | MAF, n=60 |
|---|---|---|---|---|
| chr.7:142984918 | g.4611T>C | TT | 56 | C (0.03) |
| | | TC | 4 | |
| | | CC | 0 | |
| chr.7:142985189 | g.4882G>A | GG | 59 | A (0.01) |
| | | GA | 1 | |
| | | AA | 0 | |
| chr.7:142986579 | g.6272C>G | CC | 59 | G (0.01) |
| | | CG | 1 | |
| | | GG | 0 | |
| chr.7:142986806 | g.6499G>A | GG | 59 | A (0.01) |
| | | GA | 1 | |
| | | AA | 0 | |

MAF, minor allele frequency; n, number of patients. [a]SNP (GRCh37 assembly) position in gene reference sequence NG_029248 from 5′ to 3′ end.

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**Table 4:** Significant associations of *CASP9* SNPs with its transcript levels in tumors from the PS group of breast carcinoma patients.

| Gene, SNP ID | Genotype | n | *CASP9* | *CASP9A* |
|---|---|---|---|---|
| *CASP9*, rs4645978 | GG | 12 | 1.07±0.02 | |
| | GA or AA | 48 | 1.05±0.02 | |
| | | | p=0.017[a] | |
| *CASP9*, rs4646032 | CC | 14 | 1.07±0.02 | 1.07±0.02 |
| | CT or TT | 46 | 1.05±0.02 | 1.05±0.02 |
| | | | p=0.018[a] | p=0.034 |
| *CASP9*, rs2020903 | CC | 12 | 1.07±0.02 | |
| | CT or TT | 48 | 1.05±0.02 | |
| | | | p=0.017[a] | |
| *CASP9*, rs4646034 | AA | 12 | 1.07±0.02 | |
| | AG or GG | 48 | 1.05±0.02 | |
| | | | p=0.017[a] | |
| *CASP9*, rs4661636 | GG | 24 | 1.05±0.02 | |
| | GA or AA | 36 | 1.06±0.02 | |
| | | | p=0.028[a] | |

Values of caspase expression are mean±standard deviation of the ratio of cycle threshold for a target gene to the arithmetic mean of cycle threshold of all reference genes. Therefore, the lower is the ratio the higher is the respective transcript level. p-Values were calculated by the Mann-Whitney U-test. [a]These results passed the Benjamini-Hochberg FDR test for multiple testing (q=0.029). *CASP9* haplotype is highlighted in gray.

The observed down-regulation of the initiator caspase-9 gene expression in both PS and NACT tumors could be in favor of cancer cell survival generally. Moreover, despite the lack of predictive significance of the total CASP9 transcript in NACT patients, we found association of its splicing variants (with the reported pro-apoptotic and anti-apoptotic functions) with prognosis in PS patients with breast carcinoma. Namely, the patients with a low CASP9A/B isoform expression ratio in their tumors (i.e. over-expression of anti-apoptotic CASP9B) subsequently treated with adjuvant chemotherapy had significantly shorter DFS than those with the low ratio. We have not evaluated overall survival of the followed patients due to the fact that it did not reach median. No significant difference in CASP9A/B ratio between NACT patients with partial response and poor response has been found by this study. However, we cannot exclude that this comparison is underestimated because the patients with complete response could not be included to this comparison due to the lack of tumor tissue after NACT.

CASP9B lacks a catalytic subunit and negatively affects apoptosis, probably by competitive binding to APAF1 (apoptotic protease activating factor 1, 602233), which prevents the formation of functional apoptosome [29]. Silencing of CASP9B in non-small lung carcinoma cells led to an increased sensitivity towards several types of anticancer drugs [15]. The deficiency of apoptosome activity without down-regulation of caspase-9 or Apaf-1 in ovarian cancer cell lines and primary tumors has previously been linked to chemotherapy resistance [30]. Potential role of unknown negative regulator of caspase-9 recruitment to apoptosome in this effect has been proposed, but the specific detection of CASP9B has not been performed [30]. According to the present study the CASP9A/B ratio rather than the total CASP9 transcript deserves further study.

In order to gain further functional insight into the mechanisms behind the observed association of CASP9 splicing variants with the prognosis of breast carcinoma patients, we performed a study of genetic variability of regulatory elements, including splicing sites and their proximity in the *CASP9* gene. Significant association of CASP9 transcript levels with the genetic variability in *CASP9* regulatory elements revealed by the present study is important from two points of view. First, three SNPs (rs4645978-rs2020903-rs4646034) show a high linkage disequilibrium and thus form a functionally-relevant haplotype. rs4645978 lies in the *CASP9* promoter, rs2020903 is near the splice site of *CASP9* (up to 100 bp distance), and rs4646034 is in intron 3. Secondly, the expression of progesterone receptor and ERBB2 significantly associates not only with this *CASP9* haplotype but also with the CASP9 transcript level in tumors from both studied groups of patients, suggesting a potential link to the clinical phenotype.

An influence of hormone levels on caspase 9 expression is unclear at present. Down-regulation, but also a lack of effect on the expression of caspase 9 has been described in MCF-7 breast cancer cell line after estradiol exposure in vitro [31, 32]. The down-regulation of CASP9

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**Table 5:** Significant associations of *CASP9* rs2020903 SNP with clinical data of breast carcinoma patients in the validation study (n=615).

| rs2020903 | Lymph node metastasis[a] | | OR[b] | 95% CI[b] | p-Value |
|---|---|---|---|---|---|
| | Negative | Positive | | | |
| TT | 74 | 39 | Reference | Reference | Reference |
| TC | 175 | 111 | 0.83 | 0.53–1.31 | 0.425 |
| CC | 134 | 52 | 1.36 | 0.82–2.25 | 0.233 |
| C allele | 309 | 163 | 0.99 | 0.65–1.54 | 0.997 |
| T allele[c] | 249 | 150 | **0.64** | **0.44–0.94** | **0.023** |
| | PR expression[a] | | | | |
| | Negative | Positive | | | |
| TT | 45 | 77 | Reference | Reference | Reference |
| TC | 81 | 217 | **0.64** | **0.41–0.99** | **0.050** |
| CC | 41 | 152 | **0.46** | **0.28–0.74** | **0.003**[d] |
| C allele | 122 | 369 | **0.57** | **0.37–0.86** | **0.008** |
| T allele[c] | 126 | 294 | **1.59** | **1.06–2.37** | **0.024** |
| | ERBB2 status[a] | | | | |
| | Negative | Positive | | | |
| TT | 89 | 32 | Reference | Reference | Reference |
| TC | 220 | 72 | 1.10 | 0.68–1.78 | 0.703 |
| CC | 160 | 32 | **1.80** | **1.03–3.12** | **0.038** |
| C allele | 380 | 104 | 1.31 | 0.83–2.08 | 0.243 |
| T allele[c] | 309 | 104 | **0.59** | **0.38–0.92** | **0.020** |
| | Subtype[a] | | | | |
| | TNBC | Other | | | |
| TT | 20 | 101 | Reference | Reference | Reference |
| TC | 28 | 268 | **1.89** | **1.02–3.52** | **0.043** |
| CC | 21 | 173 | 1.63 | 0.84–3.15 | 0.146 |
| C allele | 49 | 441 | **1.78** | **1.05–3.13** | **0.044** |
| T allele[c] | 48 | 369 | 0.93 | 0.54–1.61 | 0.803 |

PR, progesterone receptor; TNBC, triple negative breast carcinoma. [a]Numbers of patients in the compared groups. [b]OR, odds ratio, 95% CI=95% confidence interval. [c]CC genotype as reference. [d]This result passed the Benjamini-Hochberg FDR test for multiple testing (q=0.003). Significant results in bold.

was already observed in the other hormone-sensitive malignancies, ovarian and prostate carcinoma in comparison to non-tumor tissue [33, 34]. However, studies assessing expression levels or a prognostic relevance of CASP9 in these carcinomas are rare. G allele of the rs4645978 SNP has been associated with an increased risk of breast and pancreatic cancer [17, 35] and also with the reduced risk of lung, gastric, colorectal, prostate, and other cancers [35]. Recent meta-analysis of rs4645978 suggested the reduced prostate cancer risk in Caucasians, but not in Asians [35]. Nevertheless, the reason for the previously observed discrepancies between different tumor types remains unresolved. Authors of the recent meta-analysis suggested that heterogeneity among studies addressing these aspects might be behind such discrepancies [35].

Moreover, the in silico analysis (HaploReg v4) showed that rs2020903 SNP in *CASP9* alters the GATA and Nanog motifs with the previously suggested link to the breast carcinoma development, progression, and stemness [36, 37].

In general, the assessment of genetic variability is an important pharmacogenetic and prognostic tool and interesting cost-effective and less invasive alternative to expression profiling of tissues. However, clinical utility of each potential genetic marker should be evaluated by assessment of the functional relevance and by validation in the prospective clinical trial.

Thus, taken together our data shows that the question of clinical relevance of rs4645978-rs2020903-rs4646034 *CASP9* haplotype for cancer presents an attractive topic

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for a more detailed study on an independent set of patients and in suitable model systems.

From the other associations found, the following may attract further attention.

The previously observed over-expression of CASP2L in tumors compared with adjacent non-malignant tissues of both pre- and post-treatment patients [10] prompted us to perform analysis of genetic variability in the *CASP2L* promoter. We have identified four novel alterations in the *CASP2L* promoter and near the first exon of the *CASP2S* isoform. Functional relevance of new alterations was predicted with the help of the SNP Nexus Database (http://www.snp-nexus.org). The SNP g.4882G>A (NG_029248) lies inside a CpG island of the *CASP2L* promoter, and the g.6499G>A is located 57 bp downstream from the 3′-end of exon 1 of *CASP2S*. Due to the low MAF, the clinical importance of these alterations should be established by a larger study.

We confirmed the previously observed [38–40] over-expression of the CASP3 transcript in the PS group of patients. On the other hand, a strong down-regulation of the caspase-3 transcript and protein in tumors compared with non-malignant breast tissues was reported as well [41, 42]. The isoform-specific analysis of CASP3 (A+B and S) evaluated by the present study has shown deregulation of these isoforms to a high extent compared with the overall CASP3 transcript. Our initial protein analysis of procaspases and their cleavage products in breast carcinoma tumor samples (Supplementary Figure S2) has also shown a high variability in caspase activation and protein cleavage between samples. Thus, differences in methods used for CASP3 expression analysis, especially uncontrolled splicing transcript or protein product detection, may be among the potential reasons for the published discrepancies. Our results do not support the previously observed association of high CASP3S level with poor response to the neoadjuvant therapy in breast carcinoma patients [40].

The over-expression of CASP7 transcript in tumors expressing hormonal receptors found by the present study may be explained by the previously reported estrogen stimulation of the CASP7 expression in MCF-7 breast cancer cells in vitro [31].

Somatic mutations in caspase genes have been described in different types of carcinomas [16]. The lack of discrepancies in the distribution of alterations in the *CASP2* and *CASP9* genes between the peripheral lymphocyte and tumor DNA supports the recent finding that somatic mutation in these caspases are uncommon in breast carcinomas [43]. Comparison of germline and somatic mutation spectra in *CASP9* also strengthens the evidence for the observed association of its genotype with transcript expression.

## Conclusions

Results of this study support the hypothesis that altered expression of alternative variants of CASP9 rather than the total transcript may influence the prognosis of breast carcinoma patients. This work contributes to demonstrating the significant role of alternative splicing for cancer progression as well as the importance of detection of specific transcript variants in expression studies. Evaluation of caspase-9 pro-apoptotic activity and expression of the caspase-9B isoform in tumor samples presents a prerequisite step in the further assessment of the relevance of this alternative variant for breast cancer patients. The correlations between caspase expression levels and hormone receptor status may also have an influence on the apoptosis resistance of tumor cell in hormone receptor positive versus negative patients and how they respond to chemotherapy. Role of these caspases in the response to treatment in each breast carcinoma subgroup has not been analyzed in this study due to insufficient number of patients in the subgroups.

**Author contributions:** All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission. No writing assistance was utilized in the production of this manuscript.
**Research funding:** This work was supported by grant of the CU Grant Agency no.: 1444313 to V.B., MH CZ – DRO (National Institute of Public Health – NIPH, 75010330) project, and grant of the Internal Grant Agency of the Czech Ministry of Health, no.: 14055-3 to P.S.
**Employment or leadership:** None declared.
**Honorarium:** None declared.
**Competing interests:** The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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**Supplemental Material:** The online version of this article (DOI: 10.1515/cclm-2016-0271) offers supplementary material, available to authorized users.

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Brynychova V, Ehrlichova M, **Hlavac V**, Nemcova-Furstova V, Pecha V, Leva J, Trnkova M, Mrhalova M, Kodet R, Vrana D, Kovar J, Vaclavikova R, Gut I, Soucek P

**Genetic and functional analyses do not explain the association of high PRC1 expression with poor survival of breast carcinoma patients**

Biomed Pharmacother. 2016 Oct;83:857-864 [IF: 2,326] 6 %*)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

Biomedicine & Pharmacotherapy 83 (2016) 857–864

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# Genetic and functional analyses do not explain the association of high PRC1 expression with poor survival of breast carcinoma patients

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ARTICLE INFO

*Article history:*
Received 10 June 2016
Received in revised form 12 July 2016
Accepted 21 July 2016

*Keywords:*
Breast carcinoma
Cytokinesis
Expression
Genetic
Marker

ABSTRACT

Microtubules are vitally important for eukaryotic cell division. Therefore, we evaluated the relevance of mitotic kinesin KIF14, protein-regulating cytokinesis 1 (PRC1), and citron kinase (CIT) for the prognosis of breast carcinoma patients.

Transcript levels were assessed by quantitative real-time PCR in tissues from two independent groups of breast carcinoma patients and compared with clinical data. Tissue PRC1 protein levels were estimated using immunoblotting, and the *PRC1* tagged haplotype was analyzed in genomic DNA. A functional study was performed in MDA-MB-231 cells *in vitro*.

KIF14, PRC1, and CIT transcripts were overexpressed in tumors compared with control tissues. Tumors without expression of hormonal receptors or high-grade tumors expressed significantly higher KIF14 and PRC1 levels than hormonally-positive or low-grade tumors. Patients with high intra-tumoral PRC1 levels had significantly worse disease-free survival than patients with low levels. *PRC1* rs10520699 and rs11852999 polymorphisms were associated with PRC1 transcript levels, but not with patients' survival. Paclitaxel-induced PRC1 expression, but PRC1 knockdown did not modify the paclitaxel cytotoxicity *in vitro*.

PRC1 overexpression predicts poor disease-free survival of patients with breast carcinomas. Genetic variability of *PRC1* and the protein interaction with paclitaxel cytotoxicity do not explain this association.
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## 1. Introduction

Breast carcinoma (OMIM:114480) is the most frequent malignancy in women [1]. Therefore, it is important to identify and characterize molecular markers enabling personalized treatment and better prognostication of breast carcinoma recurrence.

Cytokinesis is the final act of the cell division and occurs immediately after mitosis [2]. Multiple mitotic kinesins and microtubule-associated proteins act together to direct cytokinesis [3]. Kinesins constitute a superfamily of microtubule-based motor proteins with diverse functions including the participation in cell division, transport of vesicles, organelles, chromosomes, protein complexes, and regulation of microtubule dynamics [4,5].

KIF14 (OMIM:611279) translocates into the positive ends of the microtubules [5] and plays an important role in mid-body formation and completion of the cytokinesis [6]. Silencing of KIF14 leads to failure of cytokinesis [7] suggesting that KIF14 is essential for this process. Overexpression of KIF14 in the 1q

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http://dx.doi.org/10.1016/j.biopha.2016.07.047
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chromosome region of genomic gain was observed in breast cancer cells [8]. The KIF14 transcript is overexpressed in tumors and predicts poor survival in breast carcinoma patients [9].

KIF14 interacts with protein regulating cytokinesis 1 (PRC1, OMIM:603484) and forms a complex with citron kinase (CIT, OMIM:605629) [6]. PRC1 promotes the formation of stable microtubule bundles during late mitosis [10] and is required for microtubule polarization and the recruitment of cytokinetic factors during monopolar cytokinesis.

CIT is Rho effector kinase that phosphorylates myosin light chain and acts as an important regulator of midbody formation during late cytokinesis [11,12]. CIT is required for KIF14 localization and its absence in the central spindle area correlates with failure of cytokinesis [6].

PRC1 has been proposed to be marker of worse prognosis in prostate, hepatocellular, and non-small lung carcinoma patients [13–15]. We recently reported the overexpression of KIF14, PRC1, and CIT in ovarian carcinomas *versus* unaffected ovarian tissues and association of intratumoral CIT levels with the time to progression of ovarian carcinoma patients ($P < 0.0001$; [16]). PRC1 (and KIF14) are grade-associated probe sets identified by PAM (Prediction Analysis of Microarrays) in breast carcinoma [17], and PRC1 was recently associated with prognosis of breast carcinoma patients [18]. However, little is known about the mechanism behind these associations. For example, genetic variability may influence gene expression and analysis of germline polymorphisms presents an attractive pharmacogenetic tool. Thus, we aimed to address this question using a haplotype-tagging approach in the most interesting candidate from the expression study.

This study explored the prognostic significance of KIF14, PRC1, and CIT gene expression in breast carcinoma patients. The previously observed association of PRC1 expression with survival of patients was validated. Therefore, its protein expression and genetic variability were characterized in breast carcinoma patients. The relevance of PRC1 in paclitaxel cytotoxicity *in vitro* was assessed to further understand its mechanism of action.

## 2. Materials and methods

### 2.1. Patients

Present study included 188 breast carcinoma patients of Caucasian origin diagnosed in The Motol University Hospital, Prague and Institute for the Care for Mother and Child, Prague between 2003 and 2010. Tumor tissues have been collected from 161 of these patients. Paired adjacent control tissues without morphological signs of carcinoma were available for 70 patients. The processing of the tissue samples was described elsewhere [19–21]. Blood samples from 99 of the above described patients were also collected. Blood samples from additional 615 patients for the genotyping validation study were collected from patients treated in The Faculty Hospital Motol, Institute for the Care for Mother and Child, The Faculty Hospital Kralovske Vinohrady (Prague, Czech Republic), and The Hospital Atlas (Zlin, Czech Republic).

Histological classification of carcinomas was performed according to standard diagnostic procedures (Supplementary Table S1). Mitotic counts were evaluated according to Elston and Ellis [22]. Post-treatment group of patients (n = 81) was treated by pre-operative regimens containing 5-fluorouracil, anthracycline, cyclophosphamide, and taxanes. Response to pre-operative therapy was evaluated by RECIST criteria [23] based on ultrasonography performed before and after the treatment. The pre-treatment group (n = 107) received adjuvant chemotherapy or hormonal therapy after surgery (for all treatments see Supplementary Table S2). Disease-free survival (DFS) was defined as the time elapsed between surgery and disease recurrence.

All patients were asked to read and sign an informed consent and the study was approved by the Ethical Commission of the National Institute of Public Health in Prague. The methods were carried out in accordance with the approved guidelines.

### 2.2. Quantitative real-time PCR

Total RNA was isolated, characterized and transcribed to cDNA as described previously [19]. Real-time PCR quantification (qPCR) of target and reference genes (Supplementary Table S3) was done essentially as previously described [19–21]. The qPCR study design adhered to the MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments; [24]).

### 2.3. Genotyping

Blood samples were collected during the diagnostic procedures using tubes with K3EDTA anticoagulant and genomic DNA was then isolated [19].

DNA sequence covering coding exons, interspersed introns, and sequences surrounding both 5′- and 3′-untranslated regions of *PRC1* gene (Chr15:89,310,277-89,338,808; NCBI Build 36.3 version) was analyzed by HaploView v4.2 program with pair-wise tagging [25]. Together 13 single nucleotide polymorphisms (SNPs) tagging common *PRC1* haplotypes at $r^2 > 0.8$ and minor allele frequency (MAF) > 0.01 in HapMap CEU data set with minimally 75% genotype data were identified (Supplementary Table S4). Additional five SNPs were included into analyses due to potentially functional relevance as miRNA binding or CpG site (Table S4). Oligonucleotide primers for these analyses were designed using the Primer3 software [26] and are available from authors upon request. SNPs were analyzed by direct DNA sequencing using Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and by high resolution melting analysis using RotorGene 6000 (Corbett Research, Sydney, Australia). Ten percent of randomly selected samples were reanalyzed with 100% concordance of results.

*PRC1* SNPs were first analyzed in a small-scale exploratory study (n = 99) and successful hits were confirmed in the large-scale independent validation study (n = 615).

### 2.4. Immunoblotting

Fresh frozen tissue samples from 17 breast carcinoma patients were available for protein expression study. Immunoblotting was performed as previously described [20,27]. Primary monoclonal antibodies against PRC1 (dilution 1:1200, catalogue no.: NB110-57434, Novus Biologicals Ltd. Cambridge, UK) or β-actin (dilution 1:1000; Sigma-Aldrich) were used. Protein bands were visualized with an enhanced chemiluminescence detection system (Thermo Scientific Pierce Protein Research Products) by Carestream Gel Logic 4000 PRO Imaging System (Carestream Health, CT, USA). Densitometry was performed using the Carestream v5.2 program (Carestream Health).

### 2.5. Functional in vitro study

Human breast carcinoma MDA-MB-231 cell line (without expression of hormonal receptors and HER2, *i.e.*, triple-negative) was used for PRC1 induction and siRNA-mediated knockdown experiments as previously described [27]. Cells were treated with 10, 30, or 100 nM paclitaxel or untreated for comparison. PRC1 transcript and protein levels were evaluated by qPCR and immunoblotting as described above. Cytotoxicity of paclitaxel was followed by cell cycle analysis using flow cytometry with flow cytometer FACSVersa (Becton, Dickinson and Company, Franklin Lakes, NJ) [28].

### 2.6. Statistical analysis

The results were evaluated by the statistical program SPSS v15.0 (SPSS, Chicago, IL) as previously described [19,27,29]. All *P*-values are departures from two-sided tests. A *P*-value lower than 0.05 was considered statistically significant. The correction for false discovery rate (FDR) was applied according to Benjamini and Hochberg [30] and q-values are provided for each comparison except survival analyses where this analysis was not performed. The functional relevance of the examined SNPs was analyzed *in silico* by Regulome DB (http://regulome.stanford.edu), Provean, and SIFT (both http://sift.jcvi.org) programs. Genetic variants and their observed associations with clinical and functional phenotype were submitted to NCBI (The National Center for Biotechnology Information) ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar) under accession numbers SCV000167256-77.

## 3. Results

### 3.1. Study population

Clinical data of breast carcinoma patients are presented in Supplementary Tables S1 and S2.

### 3.2. Differences in transcript levels between tumor and control tissues

Significantly higher transcript levels of KIF14, PRC1, and CIT were observed in tumors than controls in the post-treatment group. Significant overexpression of all three genes in tumors from the pre-treatment group of patients was also demonstrated; all results passed the FDR test (Table 1, raw data distribution in Supplementary Fig. S1).

### 3.3. Associations of transcript levels in tumors with clinical characteristics

Transcript levels of KIF14, PRC1, and CIT gradually increased with increasing tumor grade in the post-treatment group. The association for KIF14 and PRC1 in the same direction has also been observed in the pre-treatment group of patients and passed the FDR test (Table 2).

Intra-tumoral levels of all studied genes significantly associated with the expression of hormonal receptors (especially ER) in both group of patients. Associations for KIF14 and PRC1 passed the FDR test as well as those for CIT and ER (Table 2). Patients with the triple negative breast carcinoma (TNBC) subtype had significantly higher levels of KIF14, PRC1, and CIT in their tumors than the rest of the patients in the post-treatment group. PRC1 expression was associated with the TNBC subtype in the pre-treatment group as well and this association passed the FDR test (Table 2).

In survival analyses, all patients were analyzed first. Patients with higher PRC1 levels than median had worse DFS than patients with lower PRC1 levels (mean ± standard error, 82.5 ± 5.5 *vs.* 89.1 ± 4.1 months, n = 146, *P* = 0.003, Kaplan-Meier survival plot, Fig. 1). This association was also observed in a subgroup of patients treated with chemotherapy (*P* = 0.010), but not in patients treated with only hormonal therapy. Multivariate analysis using the Cox regression hazard model with pT, pN, grade, ER, PR, and HER2 as covariates confirmed the association of high PRC1 expression with poor DFS in all patients (hazard ratio, HR = 2.27, 95% confidence interval, 95% CI = 1.06-4.76, and *P* = 0.035).

The prognostic significance of PRC1 was further compared with mitotic count in histological sections (by non-parametric Kruskal-Wallis test). Gene expression of PRC1 significantly correlated with mitotic count (*P* = 0.003) although the trend was nonlinear. Unlike gene expression of PRC1, mitotic count did not influence the patients' DFS (Supplementary Fig. S2).

Transcript levels of studied genes did not significantly modify the response of patients to the neoadjuvant treatment in the post-treatment group as a whole (KIF14, *P* = 0.300; PRC1, *P* = 0.401; CIT, *P* = 0.217) nor in the subgroup of patients treated by taxane-containing regimens (KIF14, *P* = 0.651; PRC1, *P* = 0.687; CIT, *P* = 0.600; n = 39).

### 3.4. Protein levels of PRC1 in breast tumors

Further, we aimed to determine expression levels of PRC1 protein in subset of breast carcinoma and control tissues. Intra-tumoral protein levels of PRC1 were assessed by immunoblotting and normalized to β-actin as an internal control. There was a high inter-individual variability in protein levels of PRC1 among the tumor samples (mean ± standard deviation, 0.43 ± 0.30, range 0.06–1.00, n = 12; Fig. 2), but it did not correlate to the transcript ($R^2 = -0.181$, *P* = 0.539; Pearson correlation test). PRC1 was over-expressed in tumors *versus* the paired control tissue samples (0.89 ± 0.95, 0.09–3.85 *vs.* 0.25 ± 0.28, 0.01–0.68, n = 5 pairs; Supplementary Fig. S3).

### 3.5. Associations of genetic variability of PRC1 with transcript levels and clinical characteristics of patients

We also asked whether the observed PRC1 phenotype deregulation in breast carcinomas has some obvious genetic background and whether PRC1 genotype associates with clinical data of patients. Therefore, 18 SNPs tagging the haplotype of the *PRC1* gene region were genotyped in 99 breast carcinoma patients. Four additional SNPs were found in the analyzed sequences (see Supplementary Table S5 for all analyzed SNPs). MAFs of the studied SNPs did not substantially differ from HapMap-CEU population. Considering statistical power, 13 SNPs with MAF > 0.05 were used for analysis of associations with transcript levels and patients' clinical data. Associations of all SNPs with MAF > 0.05 (n = 13) with PRC1 transcript levels were analyzed, but to retain a concise style, only significant results are reported (Tables 3 and 4).

Tumors from carriers of the CC genotype in rs10520699 or the T alleles in rs11852999 expressed significantly higher PRC1 transcript levels than tumors from carriers of the T alleles in rs10520699 or CC genotype in rs11852999 (Table 3).

**Table 1**
Differences in transcript levels of studied genes between tumor and control tissues from breast carcinoma patients.

| Post-treatment group of patients (n = 81) | | | |
|---|---|---|---|
| Gene | Expression difference (fold change)[a] | Tumor *vs.* control | *P*-value[b] |
| KIF14 | 3.3 | ↑ | <0.001 |
| PRC1 | 2.1 | ↑ | <0.001 |
| CIT | 1.8 | ↑ | 0.001 |
| **Pre-treatment group of patients (n = 107)** | | | |
| Gene | Expression difference (fold change)[a] | Tumor *vs.* control | *P*-value[b] |
| KIF14 | 14.1 | ↑ | <0.001 |
| PRC1 | 5.3 | ↑ | <0.001 |
| CIT | 3.6 | ↑ | <0.001 |

↑ = overexpression in tumors compared with control tissues.
[a] Expression differences in tumors compared with control tissues calculated by the REST2009 software (Qiagen, Hildesheim, Germany).
[b] *P*-values were calculated by the Mann-Whitney test; all results passed the Benjamini-Hochberg FDR (q = 0.017).

**Table 2**
Associations of transcript levels of the examined genes in tumors with clinical data of breast carcinoma patients.

| Post-treatment group of patients | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Gene | Grade (n = 74) | | | PR expression (n = 74) | | ER expression (n = 74) | | TNBC (n = 74) | |
| | 1 | 2 | 3 | negative | positive | negative | positive | negative | positive |
| KIF14 | 1.30 ± 0.07 | 1.32 ± 0.08 | 1.24 ± 0.09 | 1.22 ± 0.08 | 1.32 ± 0.08 | 1.25 ± 0.10 | 1.30 ± 0.08 | 1.30 ± 0.09 | 1.20 ± 0.07 |
| | | ***P* = 0.001**[a] | | ***P* < 0.001**[a] | | ***P* < 0.001**[a] | | ***P* = 0.001**[a] | |
| PRC1 | 1.13 ± 0.03 | 1.10 ± 0.05 | 1.07 ± 0.06 | 1.05 ± 0.06 | 1.11 ± 0.04 | 1.06 ± 0.07 | 1.10 ± 0.05 | 1.10 ± 0.05 | 1.04 ± 0.06 |
| | | ***P* = 0.004**[a] | | ***P* = 0.004**[a] | | ***P* < 0.001**[a] | | ***P* = 0.001**[a] | |
| CIT | 1.11 ± 0.05 | 1.09 ± 0.04 | 1.07 ± 0.05 | 1.06 ± 0.05 | 1.09 ± 0.04 | 1.06 ± 0.04 | 1.09 ± 0.04 | 1.09 ± 0.04 | 1.05 ± 0.05 |
| | *P* = 0.024[a] | | | *P* = 0.018[a] | | ***P* = 0.008**[a] | | *P* = 0.020[a] | |
| **Pre-treatment group of patients** | | | | | | | | | |
| Gene | Grade (n = 84) | | | PR expression (n = 87) | | ER expression (n = 87) | | TNBC (n = 87) | |
| | 1 | 2 | 3 | negative | positive | negative | positive | negative | positive |
| KIF14 | 1.27 ± 0.08 | 1.22 ± 0.07 | 1.19 ± 0.07 | 1.18 ± 0.07 | 1.25 ± 0.07 | 1.20 ± 0.08 | 1.25 ± 0.07 | NS | |
| | | ***P* = 0.009**[a] | | ***P* = 0.005**[a] | | ***P* < 0.001**[a] | | | |
| PRC1 | 1.08 ± 0.10 | 1.03 ± 0.06 | 1.02 ± 0.05 | 1.00 ± 0.05 | 1.06 ± 0.05 | 1.01 ± 0.05 | 1.06 ± 0.06 | 1.04 ± 0.06 | 1.00 ± 0.05 |
| | | ***P* = 0.005**[a] | | ***P* < 0.001**[a] | | ***P* < 0.001**[a] | | ***P* = 0.008**[a] | |
| CIT | NS | | | 1.03 ± 0.04 | 1.05 ± 0.04 | 1.02 ± 0.04 | 1.05 ± 0.04 | 1.05 ± 0.04 | 1.02 ± 0.05 |
| | | | | *P* = 0.038[a] | | ***P* = 0.002**[a] | | *P* = 0.032[a] | |

Values are mean ± standard deviation. For analyses of associations of clinical characteristics with transcript levels in tumors, a ratio of Ct for particular target gene to arithmetic mean of Ct for all reference genes (Target gene/REF) was calculated for each sample. Therefore, the lower is the Target gene/REF ratio the higher is the respective target gene transcript level.
Associations of transcript levels with all clinical data were analyzed, but to retain a concise style only significant results replicated in both patient groups are reported. NS = Not significant.
Associations observed in both groups of patients are depicted in grey tone.
[a] *P*-values computed by the ANOVA test; results which passed the Benjamini-Hochberg FDR (q = 0.009) in bold.

Carriers of the CC genotype in rs10520699 had more frequently large (pT2-4) tumors than T allele carriers (Table 4). Patients with the T allele in rs11852999 more frequently had tumors without expression of ER and often manifested with the TNBC molecular subtype in comparison with carriers of the CC genotype (Table 4). However, none of these associations passed the FDR test.

The genetic analyses suggested that two of the 13 analyzed *PRC1* SNPs were associated with PRC1 expression and patients' clinical data. Therefore, we carried out the validation study of these two SNPs (rs10520699 and rs11852999) in an independent and larger sample of breast carcinoma patients who had clinical data for comparison (n = 615, for clinical data see Supplementary Table S6). None of the previously observed associations (Table 4) was confirmed in this validation study (Supplementary Table S7). The *PRC1* SNPs did not significantly associate with the patients' DFS either.

*In silico* analysis by Regulome DB (http://regulome.stanford.edu), Provean, and SIFT (both http://sift.jcvi.org) programs revealed that the rs10520699 SNP is located close to the binding site of transcription factors from the leucine zipper family BACH1 (OMIM: 602751), MAFF (604877), MAFK (600197), and NFE2 (601490). However, this SNP was classified as having minimal binding evidence (score 4). The same was true for the rs11852999 SNP (score 5).

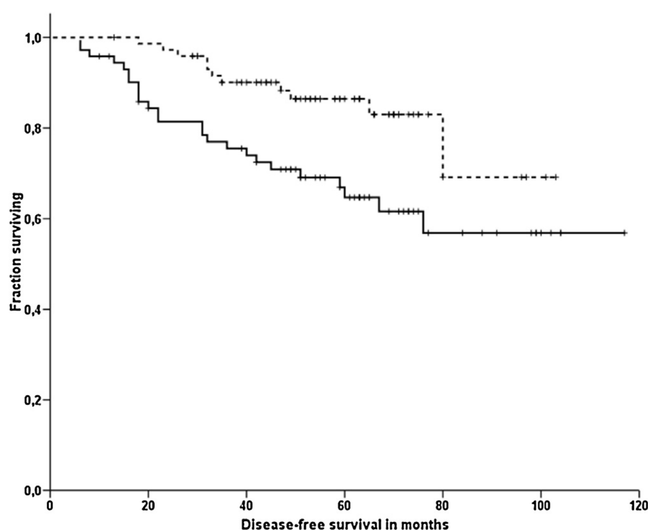


**Fig. 1.** Association between transcript levels of PRC1 in tumors and disease-free survival of all breast carcinoma patients.
Kaplan–Meier survival curves for patients with the intra-tumoral PRC1 transcript levels above the median (solid line, n = 72) *vs.* patients with the ratio below or equal the median (dashed line, n = 74) are displayed. The difference in the mean DFS between the compared groups of patients was significant (*P* = 0.003, Breslow test).

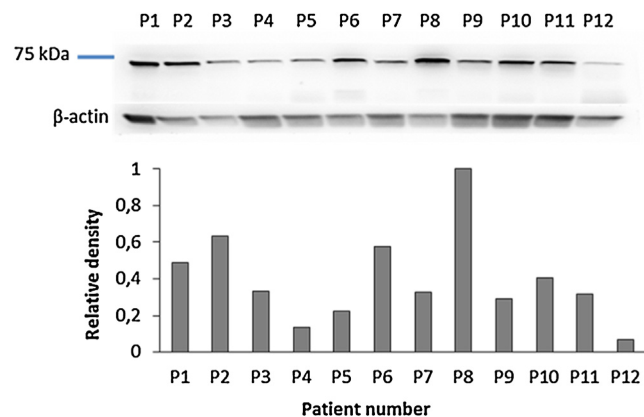


**Fig. 2.** Protein levels of PRC1 in tissues from breast carcinoma patients.
PRC1 level was assessed by immunoblotting in tumor tissues. For evaluation of the results, densitometry with normalization to β-actin was used. Cropped areas of blots represent protein bands with molecular weight of the corresponding antigen according to Human Protein Atlas (www.proteinatlas.org). kDa = kilodalton (used to describe the molecular weight of proteins), P = Patient.

**Table 3**
Association of *PRC1* gene polymorphisms with transcript levels of PRC1 in tumors from breast carcinoma patients.

| Genotype | n | Normalized PRC1 levels in tumors (Mean ± S.D.) |
|---|---|---|
| **rs10520699** | | |
| CC | 55 | 1.08 ± 0.06 |
| CT or TT | 18 | 1.11 ± 0.04 |
| Total | 73[b] | – |
| *P*-value | | 0.041[a] |
| Benjamini-Hochberg q-value | | 0.007 |
| **rs11852999** | | |
| CC | 56 | 1.10 ± 0.06 |
| CT or TT | 15 | 1.06 ± 0.06 |
| Total | 71[b] | – |
| *P*-value | | 0.026[a] |
| Benjamini-Hochberg q-value | | 0.004 |

Associations of all SNPs with MAF > 0.05 (n = 13) with PRC1 transcript levels were analyzed, but to retain a concise style only significant results are reported.
S.D.=standard deviation, n = number of patients.
[a] Analyzed by the ANOVA test. The higher is the mean the lower is the normalized PRC1 level (see Table 2 for explanation).
[b] Numbers do not add to 99 due to the missing genotypes or lack of material for PRC1 expression analysis in some patients.

**Table 4**
Associations of *PRC1* polymorphisms with clinical data of breast carcinoma patients.

| Characteristics | rs10520699[a] | | *P*-value[b] |
|---|---|---|---|
| | CC | CT or TT | 0.009 |
| pT1 | 33 | 21 | |
| pT2-4 | 34 | 5 | |
| Benjamini-Hochberg q-value | | | 0.005 |
| **Characteristics** | **rs11852999[a]** | | ***P*-value[b]** |
| | CC | CT or TT | 0.010 |
| ER-positive | 64 | 8 | |
| ER-negative | 15 | 9 | |
| Benjamini-Hochberg q-value | | | 0.005 |
| **Characteristics** | **rs11852999[a]** | | ***P*-value[b]** |
| | CC | CT or TT | 0.024 |
| TNBC | 9 | 6 | |
| Other | 70 | 11 | |
| Benjamini-Hochberg q-value | | | 0.009 |

Number of patients presented. Associations of all SNPs with MAF > 0.05 (n = 13) with all clinical data were analyzed, but to retain a concise style only significant results are reported.
pT = pathological size of tumor, ER = estrogen receptor, TNBC = triple negative breast carcinoma.
[a] Numbers do not add to 99 due to missing genotypes or clinical data.
[b] Analyzed by the Pearson's Chi Square test.

### 3.6. in vitro *functional study*

In order to evaluate potential interactions between PRC1 expression and cancer therapy outcome, the human triple-negative breast carcinoma MDA-MB-231 cell line was incubated without (control) or with 10, 30, and 100 nM paclitaxel *in vitro*. PRC1 transcript and protein levels were evaluated by qPCR and immunoblotting 12 and 24 h after addition of the drug. Paclitaxel induced slight overexpression of both transcript and protein levels of PRC1 (Fig. 3).

For analysis of taxane cytotoxicity, PRC1 knockdown was performed. 10 or 20 nM siRNA decreased cell proliferation and therefore 5 nM siRNA concentration was used (Fig. 4A). However, siRNA-directed knockdown of PRC1 transcript expression had no substantial effect on the proliferation of MDA-MB-231 cells after treatment with two G2/M block inducing concentrations (30 and 100 nM) of paclitaxel (Fig. 4B).

## 4. Discussion

Here, we followed the prognostic significance of three principal regulators of cytokinesis [6,7,11] for breast carcinoma patients. We observed strong association of PRC1 expression with DFS of the patients and subsequently examined the genetic and functional basis for this association.

The primary indication that PRC1 is a putative prognostic marker in breast carcinoma is based on several observations. First, its expression is increased more than two-fold in mammary tumors compared with non-neoplastic tissues, which is consistent with the gene expression data in Expression Atlas (https://www.ebi.ac.uk/gxa). It gradually increases with grade suggesting association with the aggressiveness of the disease. Moreover, its expression is significantly higher in tumors without expression of hormonal receptors and in tumors from TNBC patients (the worst prognosis group). These associations were found in two independent groups of patients and passed the FDR test for multiple comparisons.

Associations with clinical factors of poor prognosis are corroborated by the fact that patients with high intratumoral PRC1 levels had significantly worse DFS than those with low levels. This association is highly significant in both univariate and multivariate analyses and suggests that PRC1 is independent of clinically used prognostic factors such as stage, grade, and expression of receptors.

It is particularly interesting that PRC1 is a part of the 70-gene [31] and 64-gene [32] sets previously found to have a prognostic value in breast carcinoma patients. PRC1 (and KIF14) are among the top 264 statistically significant grade-associated probe sets identified by PAM in breast carcinoma [17]. Our data supports and further extends the previous study, which suggested that PRC1 together with other four genes (FGF18, OMIM:603726; BCL2, OMIM:151430; MMP9, OMIM:120361; and SERF1A, OMIM:603011) influences prognosis of breast carcinoma patients [18]. In contrast with the previous study using FFPE preparations [18], we used fresh frozen tissues and TaqMan assays rather than SYBR Green for qPCR analysis. Despite some methodical differences, this study confirmed PRC1 to be a putative prognostic marker in breast carcinoma patients.

Protein analysis further underlined the results of our gene expression study. The PRC1 protein overexpression in mammary tumors has already been reported [33,34], but no study addressed the correlation between PRC1 transcript and protein levels in breast carcinoma specimens so far. Although, due to the sample scarcity, the sample set for protein expression used by us is quite small, we observed no such correlation. The reasons for the lack of correlation between transcript and protein levels observed here include different normalization controls for qPCR and immunoblotting [35], posttranscriptional processing and protein stability issues. However, data on these PRC1 features is missing from the literature.

All observed associations of molecular markers with clinical data should be supported by mechanistic data. Therefore, we further asked whether the genetic variability in *PRC1* gene influences its expression and whether modulation of PRC1 expression modifies the efficacy of paclitaxel in a cell model of triple negative breast carcinoma *in vitro*.

The clinical and functional significance of genetic variation in *PRC1* was completely unknown until now. We addressed this question using a haplotype-tagging approach. Analysis of 22 SNPs covering the whole *PRC1* gene showed association of rs10520699 and rs11852999 SNPs with intra-tumoral transcript levels of PRC1. These two SNPs also significantly associated with clinical characteristics of breast carcinoma patients. We further aimed to validate our results in an independent large-scale study.

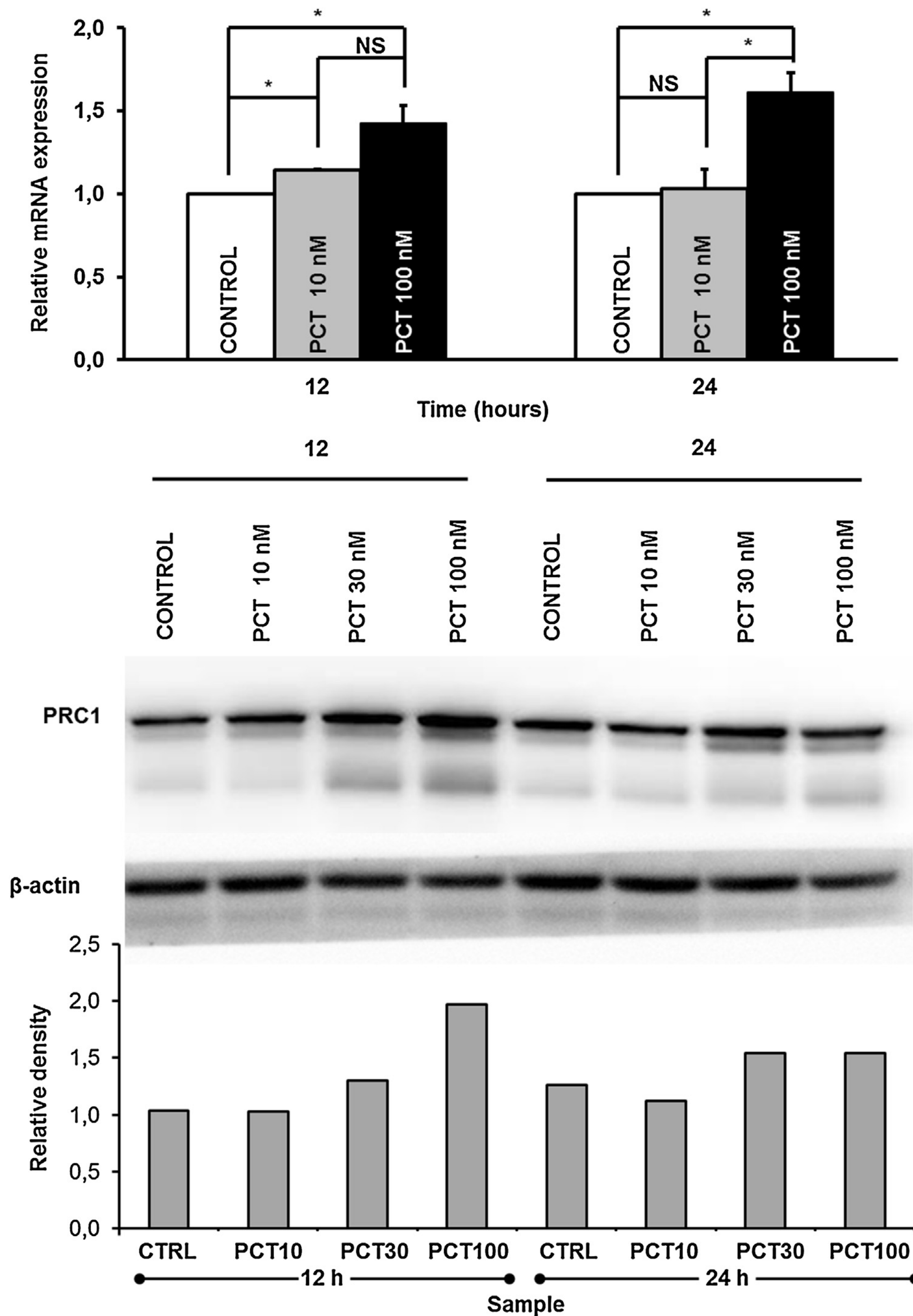


**Fig. 3.** Induction of PRC1 transcript and protein expression by paclitaxel.
Human breast carcinoma MDA-MB-231 cell line was incubated without (control) or with 10, 30, or 100 nM paclitaxel (PCT). PRC1 transcript (upper part) and protein (lower part) levels were then evaluated by qPCR and immunoblotting as described in Methods. Cropped areas of blots represent protein bands with molecular weight of the corresponding antigen according to Human Protein Atlas (www.proteinatlas.org). Two independent experiments in duplicates were performed (NS = non-significant, *P < 0.05).

However, this validation study failed to replicate the previously observed associations with clinical data, and it has not shown a significant association with patient DFS values. Thus the validation genetic study did not explain the previously suggested link between PRC1 expression, DFS, and genotype.

Moreover, we found no confirmatory data in the Catalog of Published Genome-Wide Association Studies at NHGRI (www.genome.gov) and GWAS Central (www.gwascentral.org). Additionally, no associations between PRC1 transcript levels and its SNPs have been reported by the International HapMap Project [36]. *In silico* analyses performed within the scope of the present study also showed no functional relevance of *PRC1* SNPs associated with its expression and clinical phenotypes. This data presents several lines of evidence that the observed association of PRC1 expression with DFS of breast carcinoma patients most likely has no obvious genetic background.

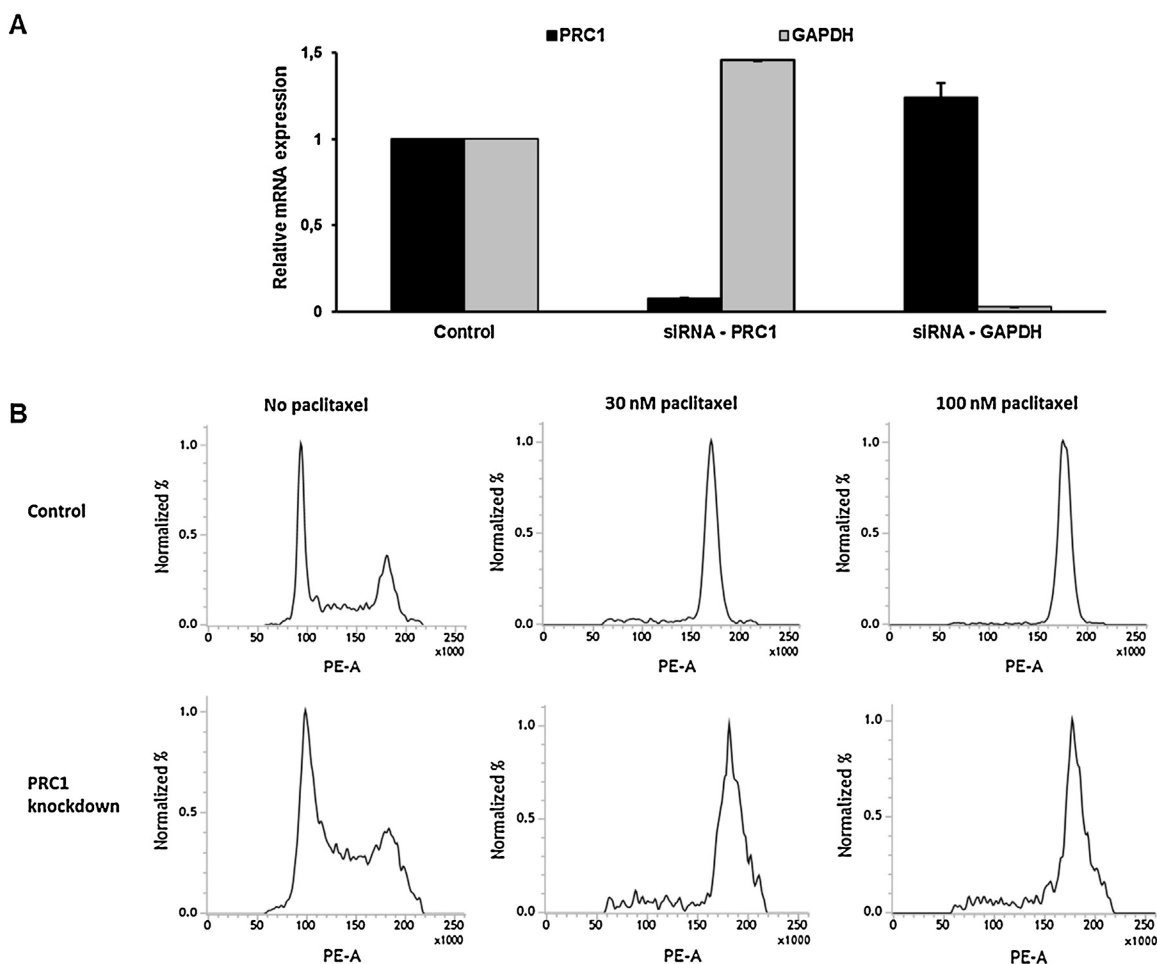


**Fig. 4.** Influence of PRC1 expression knockdown on paclitaxel cytotoxicity.
PRC1 expression was silenced by 5 nM siRNA in human breast carcinoma MDA-MB-231 cell line as documented by qPCR (a). Cells were then incubated without (control) or with 30 or 100 nM paclitaxel (PCT) and cell cycle was analyzed by flow cytometry (b) as described in Methods. Two independent experiments in duplicates were performed.

It seemed interesting to discern effects of constitutive PRC1 level from its eventual modification by anticancer drug treatment. This is challenging in *ex vivo* samples from patients due to the fact that post-treatment surgical specimens are available several weeks after last chemotherapy dose and thus eventually changed expression of the marker may level off to the pre-treatment levels. Therefore, we monitored PRC1 expression after exposure of TNBC model MDA-MB-231 human cell line to paclitaxel (frequently used in the treatment of breast carcinoma patients) *in vitro*. Paclitaxel induced both transcript and protein PRC1 levels *in vitro*. This result was surprising, because paclitaxel was clearly cytotoxic to the cells and induced formation of G2/M block, a major proof of its mechanism of action [37]. Thus, PRC1 induction by drug treatment may present one of defense mechanisms of TNBC tumor cells against paclitaxel's cytotoxicity. We thus hypothesized that PRC1 knockdown could potentiate cytotoxic effect of paclitaxel. However, siRNA-mediated knockdown of PRC1 in this model did not influence cytotoxicity of paclitaxel as a G2/M block was formed in both control and silenced cells. Thus, the above functional evidence and the fact that the response of patients to the taxane-containing regimens did not differ by the PRC1 expression demonstrate that PRC1 most probably does not modify the cytotoxic effect of taxane treatment.

Taken together, our novel data shows that the observed association of PRC1 transcript expression with DFS of breast carcinoma patients does not have plausible functional explanation. It cannot be excluded that PRC1 level just correlates with the percentage of proliferating cells that would naturally be higher in proliferating tumor cells and even higher in poorly differentiated ones in contrast to control tissue (both phenomena observed in the present study). In this case, PRC1 would be an indirect marker of proliferation without importance as predictive marker or therapy target. In our opinion, it should be further established, *e.g.*, by analysis of circulating tumor cells, whether PRC1 could serve as a marker of residual disease or expansion of aggressive tumor cell clones.

This study also confirms the previously observed overexpression of KIF14 in mammary tumors and its association with grade, but we have not found the published association of high KIF14 levels with poor DFS of breast carcinoma patients [9]. KIF14 has recently been suggested to be a putative therapeutic target in TNBC patients [38,39]. The association of KIF14 with TNBC subtype observed here agrees with the concept that further research on the role of KIF14 in the therapeutic outcome is needed specifically in TNBC patients.

CIT is overexpressed in mammary tumors and significantly associates with the ER expression of both groups of patients in the present study. Despite reports on the association of CIT with the time to progression of ovarian carcinoma [16] and its

overexpression in hepatocellular carcinoma [40], no further data on the importance of CIT for cancer progression is available.

## 5. Conclusions

This study shows that high intra-tumoral PRC1 expression is marker of poor prognosis and may be an additional prognostic classifier of breast carcinomas. Our data also shows that the observed prognostic role of PRC1 is not linked to its genetic variability and does not modify anticancer effects of paclitaxel. The study has additional possible explanations of epigenetic regulation or simple co-segregation with other molecular factors. This needs further attention.

## Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

## Acknowledgements

This work was supported by Ministry of Health of the Czech Republic, (grant No. 15-25618A to P.S.), by the National Sustainability Program I (NPU I) provided by the Ministry of Education Youth and Sports of the Czech Republic (No. LO1503 to M.E., V.B, V. H., and P.S.) and by the GAUK project (No. 1444313 to V.B.).

Authors wish to thank Dr. Anastassiya Zidkova from Institute of Biology and Medical Genetics of the First Faculty of Medicine, Charles University, Prague for STR authentication of MDA-MB-231 cell line.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2016.07.047.

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Kunicka T, Prochazka P, Krus I, Bendova P, Protivova M, Susova S, **Hlavac V**, Liska V, Novak P, Schneiderova M, Pitule P, Bruha J, Vycital O, Vodicka P, Soucek P

**Molecular profile of 5-fluorouracil pathway genes in colorectal carcinoma**

BMC Cancer. 2016 Oct 12;16(1):795 [IF: 3,265] 4 %*)

Pozn.: *) Podíl autora v % na jednotlivých aspektech díla: přípravě projektu, provádění prací, interpretaci výsledků a přípravě publikace.

BMC Cancer

RESEARCH ARTICLE Open Access

# Molecular profile of 5-fluorouracil pathway genes in colorectal carcinoma

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## Abstract

**Background:** This study addresses involvement of major 5-fluorouracil (5-FU) pathway genes in the prognosis of colorectal carcinoma patients.

**Methods:** Testing set and two validation sets comprising paired tumor and adjacent mucosa tissue samples from 151 patients were used for transcript profiling of 15 5-FU pathway genes by quantitative real-time PCR and DNA methylation profiling by high resolution melting analysis. Intratumoral molecular profiles were correlated with clinical data of patients. Protein levels of two most relevant candidate markers were assessed by immunoblotting.

**Results:** Downregulation of DPYD and upregulation of PPAT, UMPS, RRM2, and SLC29A1 transcripts were found in tumors compared to adjacent mucosa in testing and validation sets of patients. Low RRM2 transcript level significantly associated with poor response to the first-line palliative 5-FU-based chemotherapy in the testing set and with poor disease-free interval of patients in the validation set irrespective of 5-FU treatment. *UPP2* was strongly methylated while its transcript absent in both tumors and adjacent mucosa. *DPYS* methylation level was significantly higher in tumor tissues compared to adjacent mucosa samples. Low intratumoral level of *UPB1* methylation was prognostic for poor disease-free interval of the patients ($P = 0.0002$). The rest of the studied 5-FU genes were not methylated in tumors or adjacent mucosa.

**Conclusions:** The observed overexpression of several 5-FU activating genes and DPYD downregulation deduce that chemotherapy naïve colorectal tumors share favorable gene expression profile for 5-FU therapy. Low RRM2 transcript and *UPB1* methylation levels present separate poor prognosis factors for colorectal carcinoma patients and should be further investigated.

**Keywords:** Colorectal carcinoma, 5-fluorouracil, Methylation, Expression, Prognosis

## Background

Colorectal carcinoma (OMIM: 114500) is the third most common malignancy and the fourth cause of cancer-related deaths in the adult population worldwide, with the highest incidence recorded in Central Europe [1, 2].

Colorectal cancer treatment consists of surgical removal of the tumor and, based on disease characteristics, of chemo- and or radiotherapy. 5-Fluorouracil (5-FU) is widely used drug in the first-line therapy of colorectal cancer [3]. Over 80 % of administered 5-FU dose is rapidly degraded [4] and only 1–3 % is converted into its active metabolite fluorodeoxyuridine monophosphate (FdUMP [5],). FdUMP then inhibits thymidylate synthase (TYMS, OMIM: 188350) and blocks deoxythymidine triphosphate (dTTP) synthesis. Subsequent dTTP depletion triggers “thymineless” death [6]. TYMS is considered as a potential prognostic marker for colorectal cancer. Recent studies have shown that overexpression of TYMS transcript predicts poor outcome in colorectal cancer patients [7, 8]. However, another contemporary study has not confirmed these observations as intratumoral TYMS transcript level was not predictive in patients with colorectal cancer of stage II and III [9].

Several studies have indicated potential prognostic or predictive role of 5-FU metabolizing enzymes expression

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for resistance to the treatment of colorectal cancer. Colorectal cancer patients with low protein expression of 5-FU inactivating enzyme dihydropyrimidine dehydrogenase (DPYD, OMIM: 612778) exhibited a longer survival after 5-FU-treatment than those with high levels [10]. Likewise, high DPYD transcript level was associated with poor outcome of stage IV colorectal cancer patients [11]. High thymidine phosphorylase (TYMP, OMIM: 131222, 5-FU activating enzyme) transcript level was associated with significantly better disease-free survival (DFS) following oral administration of 5-FU in stage III colorectal cancer patients [12].

The resistance of the tumor cells towards 5-FU is substantially modulated by the transport mechanisms. Especially solute carrier transporter 29A1 (SLC29A1, OMIM: 602193) plays a crucial role in cellular uptake of nucleoside drugs such as cytarabine, gemcitabine, or 5-FU [13]. Results of a recent small scale functional study suggested that high SLC29A1 mRNA levels in colorectal cancer tumor tissue correlate with poor clinical response to 5-FU [14].

In this study we aimed to address importance of gene expression and methylation profile of 15 5-FU genes in tumor and adjacent bowel mucosa tissues of colorectal cancer patients for the patient's prognosis and the response to 5-FU. Genes were selected from literature and PharmGKB database based on functional evidence from 5-FU pharmacokinetics data (https://www.pharmgkb.org/). Protein expression of two most relevant candidate markers was assessed as another chain underlying 5-FU mode of action.

## Methods

### Studied patients and collection of biological specimen

Tumor tissue and adjacent non-neoplastic mucosa samples were obtained from total of 151 patients with sporadic colorectal cancer (C18-21 according to ICD-10) diagnosed at the Department of Surgery and Oncology, Teaching Hospital and Medical School in Pilsen, and General Teaching Hospital in Prague between January 2008 and November 2011. From 151 patients, 146 paired tissue samples (tumor and control mucosa), four tumors, and one mucosa sample were taken for analyses (for study flow chart, see Fig. 1). Native tissue samples were collected as described elsewhere [15, 16].

Patients represented three groups – testing set (stage II-IV, $n = 52$) for gene and protein expression and methylation analysis, validation set I (stage II, $n = 67$) for gene expression analysis, and validation set II (stage II and III, $n = 32$) for gene expression and methylation analysis. The lack of tissue aliquots for simultaneous isolation of RNA and DNA necessitated the use of two different validation sets. All patients in the testing set underwent adjuvant ($n = 26$) or palliative ($n = 26$) chemotherapy regimens based on 5-FU (with added leucovorin and/or oxaliplatin). In the validation sets I and II, 24 and 17 patients were treated by such chemotherapy regimens, respectively (Table 1 and Fig. 1).

Response to the palliative treatment was evaluated by RECIST criteria [17] based on routine imaging techniques for assessment of tumor mass (computerized tomography with or without positron emission, magnetic resonance or ultrasonography). Increase in tumor mass or the appearance of new lesions in patients with palliative treatment indicated progression and thus poor response to the treatment (PD). Good response to the treatment was defined as a decrease of the number or volume of metastases, i.e., complete or partial response (CR or PR) or stabilization of the disease or (SD). In patients treated by adjuvant therapy after radical surgical resection R0 disease-free interval (DFI) served as a measure of the treatment outcome. DFI was defined as the time elapsed between radical surgical R0 resection and disease recurrence.

Methylation analyses were conducted on 22 tissue pairs from the testing set and on the whole independent validation set II from the General Teaching Hospital, Prague.

### Isolation of total RNA and cDNA synthesis

Total RNA was isolated from frozen tissues using Trizol® reagent (Life Technologies, Carslbad, CA), stored, and characterized for the quantity and quality [18]. Complementary DNA (cDNA) was synthesized using 0.5 μg of total RNA and random hexamer primers with help of RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Quality of cDNA in terms of DNA contamination was confirmed by PCR amplification of *ubiquitin C* [19].

### Gene expression profiling

Quantitative real-time PCR (qPCR) was performed using ViiA7 Real-Time PCR System, TaqMan® Gene Expression Assays and TaqMan® Gene Expression Master Mix (Life Technologies). Reference genes - *POLR2A* (DNA-directed RNA polymerase II subunit A, OMIM: 180660), *MRPL19* (mitochondrial ribosomal protein L19, OMIM: 611832), *EIF2B1* (eukaryotic translation initiation factor 2B, subunit 1, OMIM: 606686), and *PSMC4* (proteasome 26S subunit, ATPase, 4, OMIM: 602707) - were selected by us earlier [15]. Gene Expression Assays with their characteristics are listed in Additional file 1: Table S1. While samples from the testing set were preamplified using TaqMan PreAmp Master Mix (Life Technologies), cDNA from the validation sets was used for quantification directly without preamplification procedure [20]. For calculating the qPCR efficiency of each assay, a calibration curve from one non-neoplastic sample was

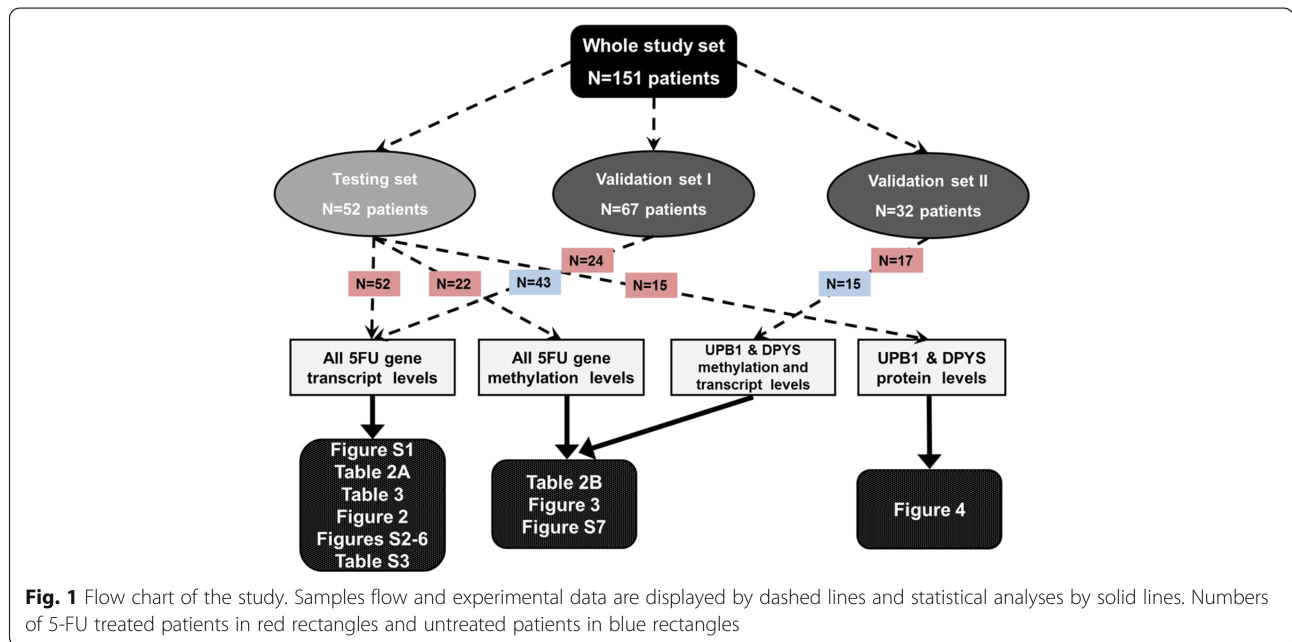


**Fig. 1** Flow chart of the study. Samples flow and experimental data are displayed by dashed lines and statistical analyses by solid lines. Numbers of 5-FU treated patients in red rectangles and untreated patients in blue rectangles

prepared (six points, 5-times dilution). The non-template control contained water instead of cDNA.

The qPCR study design adhered to the MIQE Guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments [21]).

Gene expression and clinical data of all samples were submitted to Gene Expression Omnibus (GEO) repository under accession number GSE67111.

### Promoter CpG methylation profiling

To convert unmethylated cytosines to uracils whole genomic DNA was treated with sodium bisulfite using the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Promoter region of every gene of interest was determined using Genomatix MatInspector and Genes & Genomes software (Genomatix Software GmbH, Munich, Germany). CpG islands or simple CpG sites were identified by Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA). The same software was used for design of primers specific for sodium bisulfite converted DNA bases. Number of CpGs in the PCR amplicon and equal primer melting temperature (Tm) were taken into consideration in the primer design. Real-time PCR followed by high resolution melting (HRM) was carried out in high-performance Eco Real-Time PCR system (Illumina, San Diego, CA), essentially as described in [16]. PCR was initiated by incubation at 95 °C for 5 min, followed by 50 cycles at 95 °C for 10 s, annealing temperature of specific primers (Ta) for 20 s, and 72 °C for 10 s. Primer sequences, Tm, Ta, length, and numbers of CpGs for each amplicon are listed in Additional file 1: Table S2. HRM thermal profile was set up according to the manufacturer's recommendations (Qiagen). Fluorescence data were converted into melting peaks by the Eco Software (Illumina, Ver. 3.0.16.0). For each assay, a standard dilution series of EpiTect Control DNAs (Qiagen) was run to assess the quantitative properties and sensitivity of the assay. Fluorescence of each sample was normalized against 100 % methylated DNA control. Methylation data of individual samples were subtracted from calibration curve with positive controls of 100, 75, 50, 25, and 0 % methylated DNA.

### Immunoblotting in human colorectal cancer tissues

Tissue sample pairs from 15 patients and unpaired tumors from two patients were selected based on tissue availability from the testing set and used for immunoblotting. Samples, stored at −80 °C prior to the protein isolation, were grinded by a mortar and pestle, subsequently protein and total RNA were isolated using 50 mM Tris–HCl, 150 mM NaCl, 10 % Triton X-100 buffer. Protein concentration was determined by bicinchoninic acid assay (Thermo Scientific Pierce Protein Research Products, Rockford, IL). Immunoblotting was performed as described in [20, 22]. Briefly, 10 μg of protein was used for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10 %) and transferred onto 0.2 μm Protran nitrocellulose membrane (Whatman, Kent, UK). Primary antibodies against dihydropyrimidinase (DPYS, OMIM: 613326) (dilution 1:4000), beta-ureidopropionase (UPB1 OMIM: 606673) (dilution 1:500) (both from Aviva System Biology, San Diego, CA), β-actin (dilution 1:2000; Sigma-Aldrich, St. Louis, MO) and the corresponding horseradish-peroxidase-conjugated secondary antibodies (dilution 1:10000; Sigma-Aldrich) were employed. Protein

**Table 1** Clinical-pathological characteristics of studied groups of patients

| Characteristics | Testing set ($n = 52$) | Validation set I ($n = 67$) | Validation set II ($n = 32$) |
|---|---|---|---|
| Gender (male/female) | 36/16 | 45/22 | 19/13 |
| Age at diagnosis[a] | 63.9 ± 9.2 years | 70.2 ± 9.5 years | 70.8 ± 11.2 years |
| Tumor size (pT) | | | |
| pT2 | 3 | - | 4 |
| pT3 | 40 | 62 | 24 |
| pT4 | 9 | 5 | 4 |
| Presence of lymph node metastasis (pN) | | | |
| pN0 | 15 | 67 | 18 |
| pN1-2 | 37 | - | 14 |
| Presence of distant metastasis (pM) | | | |
| pM0 | 26 | 67 | 32 |
| pM1 | 26 | - | - |
| Stage | | | |
| UICC II | 8 | 67 | 18 |
| UICC III | 18 | - | 14 |
| UICC IV | 26 | - | - |
| Histological grade (G)[b] | | | |
| GI | 6 | 9 | 6 |
| GII | 39 | 47 | 19 |
| GIII | 7 | 8 | 3 |
| Gx | - | 3 | 4 |
| Primary localization | | | |
| Colon | 26 | 44 | 28 |
| Rectosigmoideum | 12 | 9 | 1 |
| Rectum | 14 | 14 | 3 |
| Chemotherapy | | | |
| 5-FU-based | 52 | 24[c] | 17 |
| None | - | 33[c] | 15 |

Footnotes:
[a]Median ± standard deviation
[b]*GI* well differentiated, *GII* moderately differentiated, *GIII* poorly differentiated, *Gx* cannot be assessed
[c]Numbers may not add up to 67 of available subjects because of missing data ($n = 10$)

bands were visualized with an enhanced chemiluminescence detection system (Pierce Biotechnology) by Fc Odyssey (Licor Biotechnology, Lincoln, NE) and quantified by densitometry (Image Studio software, Licor Biotechnology).

### Statistical analyses

Expression levels of genes were analyzed by ViiA7 System Software (Life Technologies) and statistical analysis was performed using SPSS v16.0 Software (SPSS Inc., Chicago, IL). Fold changes were calculated usig raw cycle threshold (Ct) data by the REST2009 program (Qiagen), which is routinely used for the determination of differences between different types of sample and control groups and considers both normalization to numerous reference genes and reaction efficiencies [23]. Then ratios of Ct values of genes of interest and mean value of reference genes were calculated and used for further statistical analyses. Differences in gene expression or methylation levels between tumor and control tissues were assessed by the nonparametric Mann-Whitney U-test. To evaluate associations of transcript levels with clinical data and other variables (Table 1), nonparametric tests (the Kruskal-Wallis, the Mann-Whitney, and the Spearman's tests) were used.

DFI was evaluated by the Kaplan-Meier method and the Log Rank test was used for evaluation of the compared subgroups and combined groups of patients. Stage-adjusted analysis was performed by the Cox regression. All *P*-values were calculated from two-sided tests. *P*-values lower than 0.05 were considered statistically significant. The correction for multiple testing was applied according to Bonferroni.

## Results

### Patients' characteristics

Summary of patient's characteristics and clinical data from testing and validation sets are presented in Table 1 and the study flow diagram in Fig. 1. Testing set comprised colorectal cancer patients with stages UICC II-IV treated by first-line adjuvant ($n = 26$, UICC II and III) and palliative chemotherapy based on 5-FU ($n = 26$, UICC IV). Testing set served as a hypothesis generating screen and for assessment of protein levels. Validation set I used for validation of gene expression study included patients with UICC II stage ($n = 67$). Part of them was treated by 5-FU-based chemotherapy ($n = 24$). Validation set II used for methylation study consisted of patients with UICC II and III stage ($n = 32$) with 17 patients treated by 5-FU-based chemotherapy. The validation set II served for validation of correlations between DPYS and UPB1 methylation and expression levels and clinical data, mainly DFI. Median DFI of the validation set I was 46 ± 6 months and that of the validation set II was 39 ± 3 months.

### Transcript levels in tumors and non-neoplastic control tissues

Phosphoribosylpyrophosphate amidotransferase (PPAT, OMIM: 172450), uridine monophosphate synthetase (UMPS OMIM: 613891), ribonucleotide reductase M2 (RRM2, OMIM: 180390), and SLC29A1 transcripts were consistently overexpressed in tumors compared to adjacent mucosa in both testing and validation I sets (except UMPS, all passed the correction for multiple testing,

Table 2a, Additional file 1: Table S1). On the contrary, DPYD was downregulated in tumors compared to adjacent mucosa ($P < 0.001$, both sets).

#### Associations of transcript levels with clinical data of patients

We first tested associations between gene expression levels and therapy response of stage IV patients. Patients from the testing set with poor response to the first-line palliative treatment with 5-FU-based regimens had significantly lower expression of UMPS, ribonucleotide reductase M1 (RRM1, OMIM: 180410), and RRM2 in adjacent mucosa ($n = 26$; $P = 0.024$, $P = 0.014$, and $P = 0.038$, respectively; none passed the correction for multiple testing) than good responders (Table 3). Stage IV patients were excluded from subsequent survival analyses due to the metastatic character of their disease, which strongly modifies their prognosis.

For DFI analyses, transcript levels were first divided by their median separately in testing and validation set I and for the combined analysis these data were put together to eliminate raw data differences between sets. Significance of RRM2 gene expression for prognosis of colorectal cancer patients was further corroborated in the validation set I, where patients with intratumoral RRM2 transcript level higher than

**Table 2** Differences in transcript (A) and methylation (B) levels between tumor and adjacent mucosa tissues of colorectal cancer patients

**A. Transcript levels**

| Gene | Testing set (n=52) | | Validation set I (n=67) | |
|---|---|---|---|---|
| | Fold change [b] | *P*-value [a] | Fold change [b] | *P*-value [a] |
| | Tumor *vs* mucosa | | Tumor *vs* mucosa | |
| DPYD | 0.45[c] | <0.001 | 0.47[c] | <0.001 |
| DPYS | 0.21 | 0.014 | ND | ND |
| PPAT | 2.21[c] | <0.001 | 1.95[c] | <0.001 |
| RRM1 | 1.10 | 0.378 | 1.04 | 0.769 |
| RRM2 | 1.68[c] | <0.001 | 1.98[c] | <0.001 |
| SLC29A1 | 2.50[c] | <0.001 | 2.51[c] | <0.001 |
| TK1 | 1.64 | 0.008 | 1.27 | 0.051 |
| TYMP | 1.36 | 0.211 | 1.57[c] | 0.002 |
| TYMS | 1.20 | 0.579 | 1.29 | 0.390 |
| UCK1 | 0.86 | 0.126 | 0.75[c] | <0.001 |
| UCK2 | 1.17 | 0.355 | 1.01 | 0.680 |
| UMPS | 1.26 | 0.024 | 1.18 | 0.010 |
| UPB1 | 0.71 | 0.074 | 0.65 | 0.012 |
| UPP1 | 0.86 | 0.107 | 0.71[c] | <0.001 |
| UPP2 | ND | ND | ND | ND |

**B. Methylation levels**

| Gene | Testing set (n=22) [d] | | *P*-value | Validation set II (n=32) [d] | | *P*-value |
|---|---|---|---|---|---|---|
| | Tumor | Mucosa | | Tumor | Mucosa | |
| *DPYS* | 0.19 ± 0.14 | 0.08 ± 0.04 | <0.001[c] | 0.28 ± 0.16 | 0.20 ± 0.13 | 0.010[c] |
| *UPB1* | 0.55 ± 0.14 | 0.65 ± 0.15 | 0.022 | 0.79 ± 0.13 | 0.81 ± 0.10 | 0.436 |
| *UPP2* | 0.62 ± 0.27 | 0.56 ± 0.25 | 0.437 | ND | ND | ND |

Footnotes:
[a]Analyzed by the Mann-Whitney test
[b]Fold changes calculated by the REST2009 program
[c]Results, which passed correction for multiple testing
[d]Mean ± standard deviation of percentage of sample methylation normalized to positive control (Methods)
*ND* not determined
Results from the testing set that have been confirmed in the validation set of patients are depicted in grey

**Table 3** Differences in transcript levels in colorectal mucosa between poor and good responders to 5-FU-based chemotherapy. Transcript levels of 5-FU pathway genes were compared in mucosas of patients in the testing set divided into groups of poor responders ($n = 13$) and good responders ($n = 13$) to the first line chemotherapy regimens based on 5-FU

| Gene | Expression level in poor responders vs. good responders | | |
|---|---|---|---|
| | Fold difference[b] | Standard error[b] | *P*-value[a] |
| DPYD | 0.76 | 0.31–1.69 | 0.259 |
| DPYS | 0.91 | 0.10–6.89 | 0.434 |
| PPAT | 0.88 | 0.20–3.63 | 0.086 |
| **RRM2** | **0.31** | **0.11–1.46** | **0.038** |
| **RRM1** | **0.59** | **0.22–1.18** | **0.014** |
| SLC29A1 | 0.76 | 0.17–2.73 | 0.369 |
| TK1 | 0.87 | 0.18–3.66 | 0.157 |
| TYMP | 0.56 | 0.12–2.46 | 0.130 |
| TYMS | 0.82 | 0.15–3.77 | 0.121 |
| UCK1 | 0.85 | 0.23–1.96 | 0.369 |
| UCK2 | 0.74 | 0.17–2.42 | 0.681 |
| **UMPS** | **0.68** | **0.23–1.21** | **0.024** |
| UPB1 | 0.91 | 0.26–2.85 | 0.479 |
| UPP1 | 0.61 | 0.16–2.10 | 0.106 |

Footnotes:
[a]Analyzed by the Mann-Whitney test
[b]Fold changes and standard error calculated by the REST2009 program
Significant results in bold

median had significantly longer DFI compared to patients with levels below the median ($n = 66$, $P = 0.009$, did not pass the correction for multiple testing, Fig. 2a, the rest of results in Additional file 1: Figure S2). A non-significant association in the same direction, was observed in the testing set ($n = 26$, Additional file 1: Figure S3). Analysis of the combined testing and validation I sets supported the findings of the validation set I for RRM2 ($n = 92$, $P = 0.006$, did not pass the correction for multiple testing, Fig. 2b, the rest of results provided in Additional file 1: Figure S4). This association was significant also in stage-adjusted analysis by the Cox regression of the combined set ($n = 92$, $P = 0.013$, HR = 4.17, 95 % CI = 1.35-12.50, for all results see Additional file 1: Table S3).

Then the combined set was analyzed in respect to chemotherapy by 5-FU containing regimens ($n = 50$). However, in the combined analysis of 5-FU-treated patients from the testing and validation I sets, neither RRM2 transcript level ($P = 0.301$) nor levels of the rest of genes did significantly associate with DFI (Additional file 1: Figure S5). Stage-adjusted analysis has shown significant association between UPB1 and DFI ($P = 0.047$, HR = 0.25, 95 % CI = 0.06–0.98, for all results see Additional file 1: Table S3), which was not significant in the univariate analysis ($P = 0.098$, Additional file 1: Figure S5).

In DFI analyses of untreated patients ($n = 32$, all stage II from the validation set 1), low level of UPB1 ($P = 0.026$, did not pass the correction for multiple testing) and TYMP ($P = 0.047$, did not pass the correction for multiple testing) significantly associated with worse DFI of patients (Additional file 1: Figure S6).

### Methylation levels in tumors and non-malignant adjacent mucosa, associations with gene expression, and clinical characteristics

Methylation of CpG islands in the regulatory regions of all studied genes was initially studied in 22 pairs of tumor and adjacent mucosa (testing set) and compared with that from the independent validation set II. In the both testing and validation II sets, methylation exceeding the limit of quantitation was detected in *DPYS*, *UPB1*, and uridine phosphorylase (*UPP2*, GeneID: 151531) genes in both tumor and adjacent mucosa samples (Table 2b, DPYS passed the correction for multiple testing). Significantly elevated methylation level of *DPYS* was recorded in tumor tissues compared to adjacent mucosa in both sets (Table 2b). Methylation level of *UPB1* was lower in tumors than in adjacent mucosa in the testing set, but not in the validation set II. No difference in promoter methylation was observed for *UPP2* in the testing set by comparing tumors with non-malignant mucosa.

Methylation levels in promoter regions of *DPYS* or *UPB1* did not correlate with their corresponding transcript levels either in tumors or in adjacent mucosa samples analyzed in both sets. UPP2 transcript expression was below the limit of quantification in both testing and validation II sets suggesting that this gene is completely silenced in colorectal tumors and corresponding adjacent mucosa tissues regardless clinical characteristics.

*DPYS* methylation level was associated with the tumor stage in the testing set ($P = 0.010$, data not shown), but not in the validation set II. Therefore, this association is not further discussed. On the other hand, patients with *UPB1* methylation level below the median had significantly worse DFI than those with the methylation level above the median in both sets evaluated separately (Additional file 1: Figure S7) and combined ($n = 46$, $P = 0.0002$, passed the correction for multiple testing, Fig. 3). This association was significant also in the stage-adjusted analysis by Cox regression of the combined set ($n = 46$, $P = 0.004$, HR = 9.22, 95 % CI = 2.04-41.57).

Combined analysis of *UPB1* methylation in 5-FU treated patients from testing and validation II sets failed to find significant association with DFI ($n = 32$, $P = 0.653$, data not shown). For DFI analyses, patients were divided into two groups according to the median of methylation levels in tumors. Methylation levels of *DPYS* and *UPP2* have not associated with the DFI of patients ($P > 0.05$).

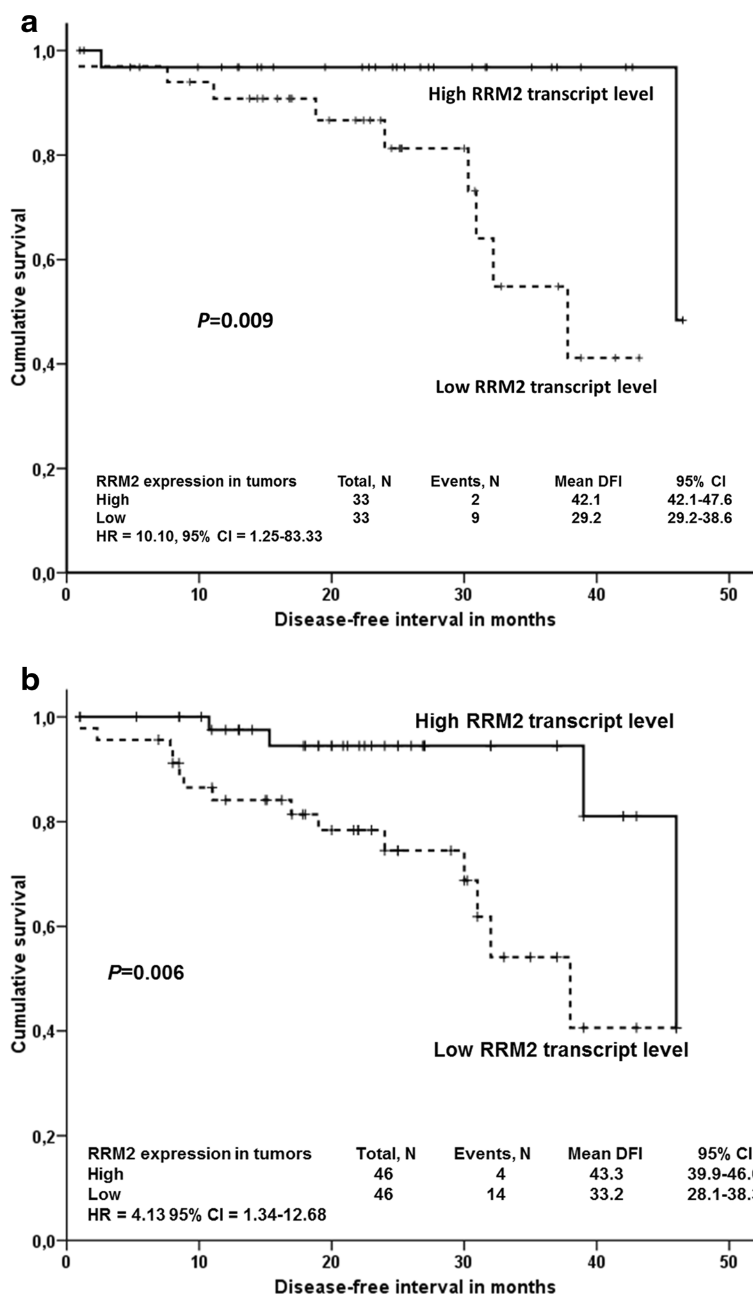


**Fig. 2** Association between RRM2 transcript levels and DFI of colorectal cancer patients. Kaplan-Meier survival curves were plotted for patients ($n = 66$, one patient was lost to follow up) from the validation set I (**a**) or combined testing and validation I sets ($n = 92$) (**b**). Patients were divided into two groups according to the median of transcript levels in tumors. Dashed line represents the group with lower transcript levels, solid line the group with higher transcript levels than median. Differences between groups were compared using Log-rank test. All genes have been analyzed, but to retain concise style only significant association is reported. HR = hazard ratio, 95 % CI = 95 % confidence intervals for stage-adjusted analyses

### Protein levels in tumors and adjacent non-malignant mucosa

DPYS and UPB1 protein levels were analyzed in a subset of the testing set used for the methylation study, enabling an evaluation of the cascade of methylation, gene, and protein expression levels in colorectal cancer samples (Fig. 4). However, DPYS and UPB1 protein levels did not significantly correlate either with their transcripts or methylation levels ($P > 0.05$).

## Discussion

The questions connected with prognostic importance of molecular profile of 5-FU pathway in colorectal cancer remain attractive topics throughout last 15 years. Existing

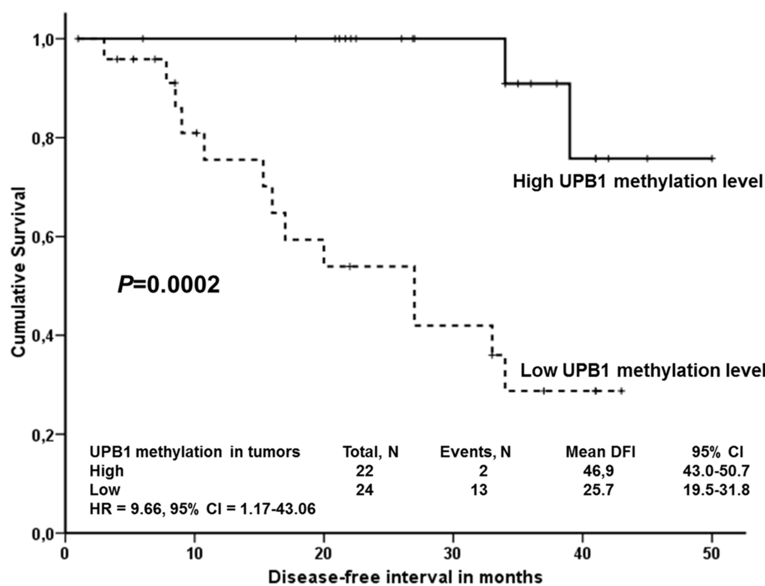


**Fig. 3** Association between *UPB1* methylation levels and DFI of colorectal cancer patients. Kaplan-Meier survival curves were plotted for patients from the both testing and validation II sets combined ($n = 46$). Seven stage IV patients were excluded and for further 31 patients data on methylation or DFI were not available. Patients were divided into two groups according to the median of intratumoral gene methylation levels. Dashed lines represent the group with lower methylation levels and *solid lines* represent the group with higher levels than median. Differences between these groups were compared using Log-rank test. HR = hazard ratio, 95 % CI = 95 % confidence intervals for stage-adjusted analyses

studies offered a plethora of mostly conflicting results. The absence of complex understanding, focused on mechanisms of action underlying the most promising biomarkers precludes their translation into clinical setting. Apparently, the final prognostic scheme will integrate clinical factors, e.g., stage and grade of the tumor with a cascade of molecular markers involving genetic, epigenetic, and phenotypic factors. The present study brings completely new insight into this area by comprehensive molecular profiling of major 5-FU pathway genes.

The present study shows for the first time that only three (*DPYS*, *UPB1*, and *UPP2*) out of 15 evaluated 5-

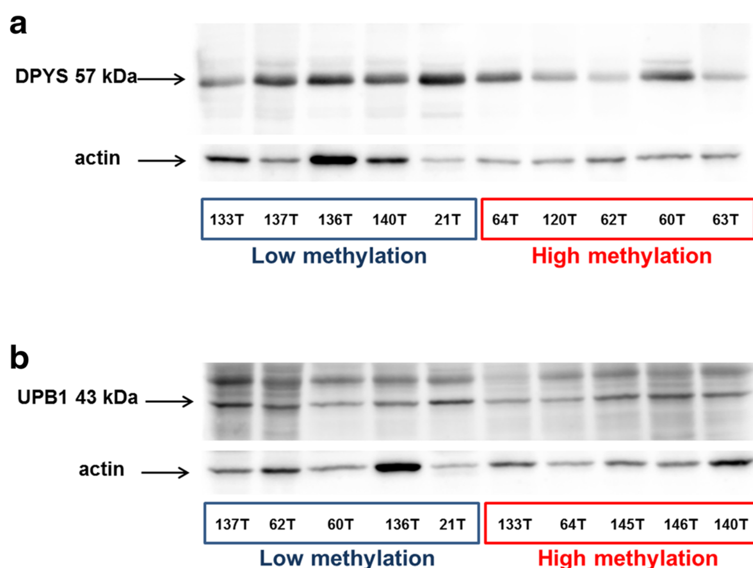


**Fig. 4** Protein expression of DPYS and UPB1 in tumors of colorectal cancer patients. Protein expression of DPYS (**a**) and UPB1 (**b**) was assessed by immunoblotting with normalization to actin in the representative set of tumors with highest and lowest methylation levels as described in Materials and Methods

FU genes, are subject to notable methylation in tumor and adjacent mucosa tissues.

Association of *UPB1* promoter methylation with worse prognosis of colorectal cancer patients, reported here on two independent groups of patients and in the combined set irrespective of 5-FU treatment, poses a completely novel direction in pharmacogenomics of colorectal cancer. UPB1 is an 5-FU inactivating enzyme [24], responsible for degradation of pyrimidine bases (uracil and thymine) and its genetic defect causes severe forms of propionic acidemia [25]. We hypothesized that a high UPB1 expression in tumor cells caused by promoter demethylation could exert a negative impact on the colorectal cancer patients response to 5-FU. However, we did not prove such association in the combined set of 5-FU treated patients and moreover, *UPB1* methylation level did not correlate with either the transcript or the protein levels suggesting that its prognostic role is most probably a complex phenomenon involving some other factors. The lack of such correlation may be explained by a number of effects, e.g., variation in DNA folding in the studied region, regulation of target gene by enhancers/silencers or by other than the followed CpGs or control of gene expression by histone modifications. A more refined screening of CpG methylation in the *UPB1* surrounding area could provide more information about potentially linked epigenetic changes. Moreover, the function of the above mentioned gene may also be modulated by microRNA interference (e.g., hsa-miR-216a, predicted by TargetScan).

From the genetic point of view it is intriguing that recent study reported a strong association between the rs2070474 polymorphism and gastrointestinal toxicity in 5-FU treated cancer patients [26]. It is of interest that this polymorphism lies inside a large CpG island consisting of 98 CpG sites [27] and near to the transcription factor-binding motifs corresponding to a critical regulator of the intestine, the CDX2 (caudal-type homeobox transcription factor 2, OMIM: 600297 [28];). A potential linkage of genetic with epigenetic changes thus should also be considered.

On the basis of our gene expression data we may generalize, that colorectal tumors irrespective of the stage and localization share common downregulation of DPYD and upregulation of PPAT, UMPS, RRM2, and SLC29A1 transcripts. RRM2 and UMPS upregulations and DPYD downregulation in colorectal tumors comply with the previous study [29].

Interestingly, SLC29A1 was recently suggested as potential co-determinant of clinical response to 5-FU [14] and its upregulation demonstrated by the present study further underpins the potential for targeted therapy of colorectal cancer. On the basis of gene expression profile we may deduce that chemotherapy-naïve colorectal cancer patients have in general favorable expression profile shifted towards 5-FU activation (Fig. 5). A potential change of this profile by chemotherapy or during metastatic process presents another interesting question that needs to be addressed.

Moreover, promoter of the 5-FU inactivating enzyme DPYS was found hypermethylated in colorectal tumors by this study. We thus confirmed the previously published *DPYS* hypermethylation in colon carcinomas (and breast and prostate carcinomas) compared with paired normal tissues from the same patients [30]. Recently, it

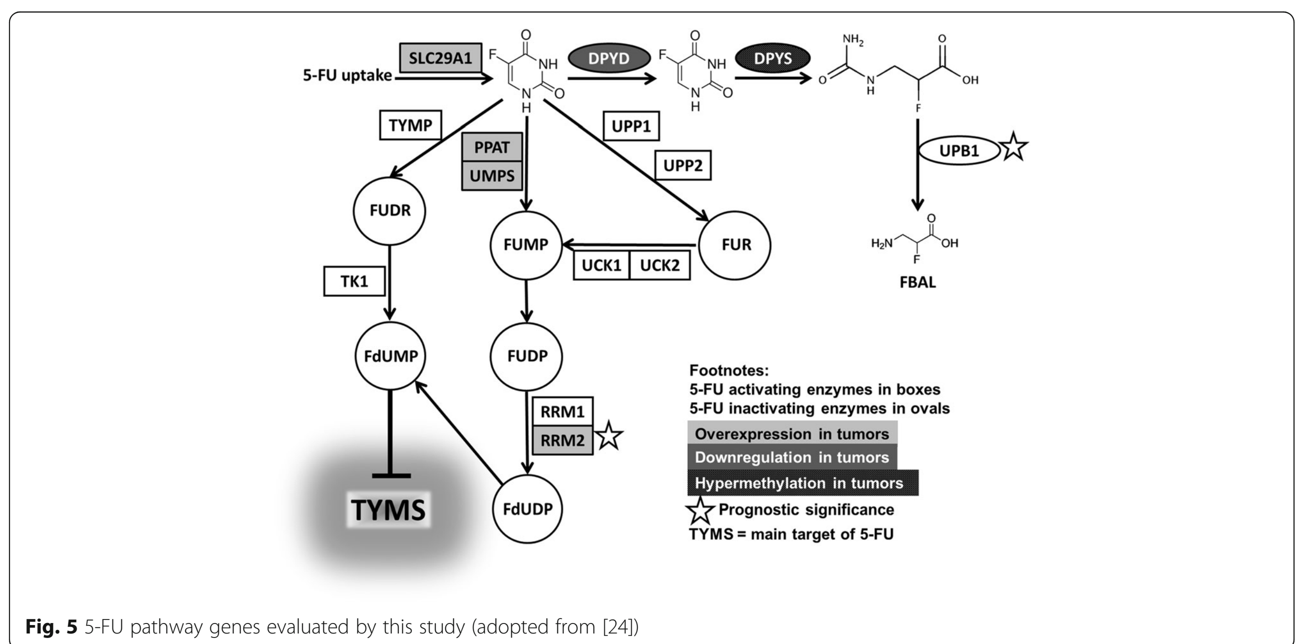


**Fig. 5** 5-FU pathway genes evaluated by this study (adopted from [24])

was reported that differential methylation of *DPYS* (and heat shock 27 kDa protein 1, *HSPB1*, OMIM: 602195 and cyclin D2, *CCND2*, OMIM: 123833) provides independent prognostic information for prostate carcinoma [31]. Based on the present and earlier studies, colorectal cancer-specific complex prognostic model based on gene expression and methylation profile seems to deserve further exploration.

Prognostic significance of low RRM2 transcript level for poor colorectal cancer patient's outcome observed by the present study contradicts the previously published data. High RRM2 level was poor survival predictor in colorectal cancer patients [32] reflecting the established in vitro ability of RRM2 to enhance cellular invasiveness and genetic instability [33]. We cannot rule out that the qPCR assay for RRM2 employed in the present study also covered the RRM2B (OMIM: 604712) subunit whose protein structure is 80 % identical to RRM2. RRM2B intriguingly exerts opposite activity to RRM2 and its expression associates with a better survival of colorectal cancer patients [34]. On the other hand, RRM2 is 5-FU activating enzyme [24] and thus the result observed by us seems logical from this point of view despite the fact that we have not observed a direct link between prognostic role of RRM2 and 5-FU therapy (perhaps due to the low number of the followed patients). Bearing in mind the issue of study size and publicly available gene expression data, we analyzed the prognostic power of RRM2 expression by SurvExpress [35] tool using data from GSE12945 set ($n = 947$). A borderline significant association towards higher risk of shorter disease-free survival of the patients with lower expression of RRM2 was apparent ($p = 0.050$, Additional file 1: Figure S8).

The present study in line with other authors [9], has not confirmed that overexpression of TYMS protein or transcript predicts poor outcome in colorectal cancer patients [7, 8]. Similarly, the results of studies indicating potential prognostic role of DPYD [10, 11] or TYMP [12] expression for survival of colorectal cancer patients after 5-FU-treatment were not replicated.

The small sample size and small patient's groups used for DFI analyses, especially of patients treated with 5-FU pose the major limitations of this study. Nevertheless, we compared the methylation profiles with the publicly available database MethHC (Methylation and gene expression in Human Cancer, http://methhc.mbc.nctu.edu.tw) integrating gene expression, methylation, and microRNA expression data from The Cancer Genome Atlas (TCGA) [36]. Our data complies with the results reported by this database, i.e., the highest levels in *UPP2*, *UPB1*, and *DPYS* (the rest of the genes below 25 %) and significantly higher methylation of *DPYS* in tumor compared with mucosa tissues (Additional file 1: Figure S9).

The variability among the patient cohorts could also explain the lack of replication of some results. On the other hand, the use of validation sets helped to achieve more convincing interpretation of the replicated results and where possible the analysis of combined sets increased the study power. The lack of tissue aliquots for simultaneous isolation of RNA and DNA necessitated the use of two different validation sets. This fact precluded us to perform the otherwise preferable combined analyses of both validation sets. Consequently, missing data for comparison of methylation levels with DFI may be seen as a study limitation.

## Conclusions

In this study, we addressed importance of genes involved in the 5-FU pathway for the prognosis of colorectal cancer patients. In conclusion, chemotherapy-naïve colorectal tumors seem to have favorable 5-FU pathway gene expression profile. Additionally, low RRM2 gene expression and *UPB1* methylation level represent treatment-independent poor prognostic factors for colorectal carcinoma patients and should be further investigated in relation to other epigenetic regulation pathways (such as microRNAs) and in a complexity with other relevant systems, such as DNA repair.

## Additional file

**Additional file 1: Table S1.** Lists TaqMan Gene Expression Assays used in the study. **Table S2** shows sequence of primers and PCR conditions used for promoter CpG methylation profiling. **Table S3** shows results of stage-adjusted Cox regression of associations between transcript levels and DFI of colorectal cancer patients from the combined testing and validation I sets. **Figure S1** depicts 5-Fluorouracil pathway gene expression levels in the studied sets of colorectal cancer patients. **Figure S2** shows results of analysis of associations between transcript levels and disease-free survival of colorectal cancer patients from the validation set I. **Figure S3** shows results of analysis of associations between transcript levels and disease-free survival of colorectal cancer patients from the testing set. **Figure S4** shows results of analysis of associations between transcript levels and disease-free survival of colorectal cancer patients from the combined testing and validation I set. **Figure S5** shows results of analysis of associations between transcript levels and disease-free survival of 5-fluorouracil-treated colorectal cancer patients from the combined testing and validation I set. **Figure S6** shows results of analysis of associations between transcript levels and disease-free survival of untreated colorectal cancer patients from the validation I set. **Figure S7** shows results of analysis of associations between *UPB1* methylation levels and disease-free survival of colorectal cancer patients. **Figure S8** shows analysis of association of RRM2 expression with disease-free survival of colorectal cancer patients based on publicly available GEO database. **Figure S9** shows analysis of methylation profiles of 5-FU pathway genes in human colorectal tumor (red boxes) and mucosa (green boxes) tissues from publicly available MethHC database. (DOC 1916 kb)

### Abbreviations

5-FU: 5-fluorouracil; 95 % CI: 95 % confidence interval; CCND2: Cyclin D2; cDNA: Complementary DNA; CDX2: Caudal-type homeobox transcription factor 2; CpG: Cytosine-phosphate-guanine; CR: Complete response; DFI: Disease-free interval; DFS: Disease-free survival; DPYD: Dihydropyrimidine

dehydrogenase; DPYS: Dihydropyrimidinase; dTTP: Deoxythymidine triphosphate; EIF2B1: Eukaryotic translation initiation factor 2B, subunit 1; FDR: False discovery rate; FdUMP: Fluorodeoxyuridine monophosphate; GEO: Gene expression omnibus; HR: Hazard ratio; HRM: High resolution melting; HSPB1: Heat shock 27kDa protein 1; ICD-10: The international classification of diseases, version 10; MIQE: Minimum information for publication of qPCR experiments; MRPL19: Mitochondrial ribosomal protein L19; OMIM: Online mendelian inheritance in man; PCR: Polymerase chain reaction; PD: Progression of the disease; PharmGKB: The pharmacogenomics knowledgebase; POLR2A: DNA-directed RNA polymerase II subunit A; PPAT: Phosphoribosylpyrophosphate amidotransferase; PR: Partial response; PSMC4: Proteasome (prosome, macropain) 26S subunit, ATPase, 4; qPCR: Quantitative real-time PCR; RECIST: Response evaluation criteria in solid tumors; RRM1/2: Ribonuclease reductase subunit M1/2; SD: Stabilization of the disease; SLC29A1: Solute carrier transporter 29A1; Ta: Annealing temperature; TK1: Thymidine kinase; Tm: Melting temperature; TYMP: Thymidine phosphorylase; TYMS: Thymidylate synthase; UCK1/2: Uridine cytidine kinase 1/2; UICC: Union for international cancer control; UMPS: Uridine monophosphate synthetase; UPB1: Beta-ureidopropionase; UPP1/2: Uridine phosphorylase 1/2

**Acknowledgements**
Not applicable.

**Funding**
This work was supported by the Internal Grant Agency of the Czech Ministry of Health (project no.: NT/14329-3 to V.L.), Czech Science Foundation (project no.: P301/12/1585 to P.V.), Grant Agency of Charles University in Prague (project no. 1200314 to T.K.), and by the National Sustainability Program I (NPU I) (project no. LO1503 to P.S.).

**Availability of data and materials**
The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

**Authors' contributions**
TK, PaP, IK, PB, PM, SS, and HV carried out the experimental studies, participated in the evaluation of results, and drafted the manuscript. PS, VL, and PP designed and coordinated the study, performed statistical analyses, and drafted the manuscript. NP, SM, PiP, BJ and VO recruited patients, collected clinical data, and drafted the manuscript. All authors read and approved the final manuscript.

**Authors' information**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

**Consent for publication**
Not applicable.

**Ethics approval and consent to participate**
All patients, aware of the study aims, methods and potential risks, signed consensually an informed consent form. The study was approved by the Ethical Committees of the Medical Faculty and Teaching Hospital in Pilsen and General Teaching Hospital in Prague, Czech Republic.

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Received: 4 January 2016 Accepted: 30 September 2016
Published online: 12 October 2016

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**Importance of transcript levels of caspase-2 isoforms S and L for breast carcinoma progression**

Future Oncol. 2013 Mar;9(3):427-38 [IF: 3,202] 4 % *)

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# Importance of transcript levels of caspase-2 isoforms S and L for breast carcinoma progression

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**Aim:** A role of caspase-2 in chemotherapy-induced apoptosis has been suggested. Our study aimed to evaluate the prognostic and predictive importance of caspase-2 isoforms in breast cancer patients. **Materials & methods:** Caspase-2L and -2S transcript levels were determined in paired tumor and non-malignant control tissues from 64 patients after neoadjuvant chemotherapy and 100 pretreatment patients (general set) by real-time PCR with absolute quantification. **Results:** Low but statistically significant upregulation of caspase-2L in tumor versus control tissues was observed in both sets. Significant associations of the levels of caspase-2L, -2S or S/L ratio with clinical prognostic factors were observed. However, none of these associations were confirmed in both sets. Levels of caspase-2 isoforms or the S/L ratio did not significantly associate with progression-free survival in the general set or with chemotherapy response in the neoadjuvant set. **Conclusion:** Our results suggest that the role of caspase-2 isoforms in the progression of breast cancer may considerably differ between pre- and post-chemotherapy patients.

Anthracyclines and taxanes are among the most frequently used drugs for treatment of hormonally nonresponsive breast carcinomas in the adjuvant (post-operative) or neoadjuvant (preoperative) setting. Taxanes inhibit tubulin depolymerization, causing arrest of cells in mitosis. Apoptosis and mitotic exit are two possible fates following mitotic arrest [1].

Researchers have intensively studied taxane effects in tumor cell models. Both classical (paclitaxel and docetaxel) and novel (Stony Brook) taxanes have been involved in the induction of apoptosis in these models [2–4]. Comparing resistant (NCI/ADR-RES) versus sensitive (MDA-MB-435) tumor cells, it has been shown that pathways of apoptosis induction by taxanes could differ in certain key events; for example, $G_2$/M block, the release of cytochrome c from mitochondria and activation of caspases [2,3]. A key role of caspase-2 [101] in apoptosis induced by taxanes has been suggested by the current authors [4] and others [5,6]. Moreover, a high level of caspase-2 was associated with a lower survival probability in patients with acute lymphoblastic leukemia [7]. Caspases are cysteine-dependent aspartate-directed proteases that play an essential role in the execution phase of apoptosis. Caspase-2 (also known as ICH-1 or NEDD2) is a unique member of its family because it shares features with both initiator and effector caspases [8]. Caspase-2 is activated in response to various apoptotic stimuli and increasing lines of evidence indicate the nonapoptotic roles of caspases; for example, DNA repair and cell cycle regulation [8]. A tumor-suppressor function for caspase-2 has also been described [9,10].

Alternative splicing of caspase-2 transcript leads to two mRNA species encoding two proteins (i.e., a long isoform caspase-2L and a short isoform caspase-2S) that have antagonistic effects on apoptosis [11,12]. A 100-nucleotide sequence element, named In100, inside intron 9

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Keywords
■ breast cancer ■ caspase-2 ■ prognosis ■ transcript

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represses the inclusion of alternative exon 9 into the caspase-2L mRNA [13]. The inclusion of the 61 bp exon leads to caspase-2S mRNA with a premature stop codon in exon 10 due to the frameshift. This mechanism appears to be universal and generation of enzymatically active versus inactive isoforms by alternative splicing appears to provide an excellent mechanism for fine-tuning the level of active caspases at the post-transcriptional level [14].

The aim of our study was to: explore transcript levels of caspase-2L and -2S isoforms in tumor and non-malignant control tissues from breast cancer patients treated with anticancer drugs, evaluate associations of transcript levels of caspase-2L, -2S and the caspase-2L/-2S (S/L) ratio with clinical prognostic factors and elucidate the implication of caspase-2 transcripts in tumors for chemotherapy efficacy. Importance of genetic variability in the In100 regulatory element and adjacent exonic and intronic sequences for expression of caspase-2 transcripts *in vivo* was also addressed. To date none of these aspects have been studied in breast cancer patients.

## Material & methods

### Subjects

In total, 164 samples of mammary carcinomas and 81 paired adjacent non-malignant tissues (controls) without morphological signs of carcinoma were obtained from incident breast cancer patients. Cases were consecutively diagnosed in two hospitals in Prague during the periods between February 2003 and December 2007 (general set, n = 100; Faculty Hospital in Motol [Prague, Czech Republic] – described in detail in [15]), and between July 2007 and March 2009 (neoadjuvant set, n = 64; Medicon Hospital [Prague, Czech Republic] – described in detail in [16]). Blood samples from 83 patients under study were also collected. The processing of samples is described in the Supplementary Material (see online at www.futuremedicine.com/doi/suppl/12.200). All patients were asked to read and sign an informed consent form in accordance with the requirements of the Ethical Commission of the National Institute of Public Health in Prague.

### DNA isolation & mutation analysis

Blood samples were collected during the diagnostic procedures using tubes with K3EDTA anticoagulant. Genomic DNA was isolated from peripheral blood lymphocytes by the phenol/chloroform extraction method [16]. Presence of alterations in the caspase-2 region containing exons 8–10, splicing regulatory element In100 and adjacent intronic sequences was determined by DNA sequencing (see Supplementary Material for detailed protocol).

### Total RNA & cDNA preparation

Tissue sections (10–20 pieces of 5 µm sections) from the general set were directly transferred to 1 ml of Trizol® reagent (Invitrogen, CA, USA). Frozen tissues (~2 × 2 × 2 mm blocks) from the neoadjuvant set were first homogenized by mechanical disruption using a Precellys instrument (Bertin Technologies, Montigny-le-Bretonneux, France) at a speed of 6500 rpm for 15 s. Total RNA was isolated from all samples using Trizol reagent according to the procedure supplied by the manufacturer and stored in 20 µl aliquots at -80°C. No DNAse I treatment of total RNA was performed. Quant-iT RiboGreen RNA Quantitation Assay Kit (Invitrogen) was used to determine the total RNA concentration of the samples in duplicate. The RNA quality was assessed by measuring the RNA Integrity Number (RIN) using Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Assay Kit by Agilent Technologies, Inc. (CA, USA). The RIN value was in range (2.3–8.9) and 6.5 on average. More than 95% of all samples had a RIN over 3. cDNA was synthesized using 0.5 µg of total RNA using random hexamer primers with the help of RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). The quality of cDNA, in terms of DNA contamination, was then confirmed by PCR amplification of ubiquitin C fragment discriminating between the product from cDNA (190 bp) and genomic DNA (1009 bp) as described [17]. All cDNA samples that were free of DNA contamination were further analyzed.

### Construction of standards for absolute quantification

Bacterial plasmids containing the respective target sequences for TaqMan Assays of caspase-2L, caspase-2S and *MRPL19* reference gene were used as standards for absolute quantification of gene expressions by real-time PCR. For this purpose, PCR fragments were prepared with the following primers: caspase-2S (see the primers for real-time PCR), caspase-2L (forward: 5′-AGCTCTTTGACAACGCCAA-3′ and reverse: 5′CAGGAACCTCGTTTGGTGTT-3′) and *MRPL19* (forward: 5′-GTGAGCTAGCTGGCATGG-3′ and reverse: 5′-TTCGACGCTTCAATTTCCTT-3′, all from Sigma-Aldrich, MI, USA), and were subcloned

into the vector pDONR201 using gateway cloning technology, as previously described [15]. Plasmids were serially diluted in nuclease-free water (from $10^6$ to $10^2$ copies per reaction) for construction of the calibration curve.

### Quantification of transcript levels by real-time PCR

TaqMan Gene Expression Assays (Life Technologies, CA, USA) were used for detection of transcript levels of caspase-2L isoform (Hs00895082_m1) and reference gene *MRPL19* (Hs00608519_m1). *MRPL19* for normalization of *CASP2L* and *S* levels was chosen based on its highest stability among 11 genes evaluated by quantitative PCR in benign and malignant primary breast cancer tissues [18]. For the detection of caspase-2S transcript a specific assay was designed. Primers and a probe were designed using Primer3 software [102] and checked for specificity by BLAST [103]. Primers or probes for quantitative PCR spanned exon–exon boundaries to avoid amplification of genomic DNA. The sequences of primers and the probe for caspase-2S transcript assessment (165 bp amplicon length) were forward: 5′-CCGTGGAGGTGCTATTGG-3′, reverse: 5′-TCGGCAACTTTTCTTTACCG-3′ and probe: 5′-FAM-GTTCACTGCTGCCACC-GCCT-NQF-3′ (Life Technologies). Specificity of caspase-2L and -2S assays was tested by real-time PCR quantification in cross reactions containing serially diluted plasmid standards. Both assays were specific above 50 copies per reaction and, thus, the limit of quantification was set to 100. Real-time PCR quantification was carried out on ViiA™ 7 Real-Time PCR System (Life Technologies) in a 5 µl reaction mixture containing 1 × TaqMan® Universal PCR Master Mix (Life Technologies) and 1 × TaqMan Gene Expression Assay (Life Technologies) or 500 nM each of primers and probe (caspase-2S) and 2 µl of diluted cDNA. Cycling parameters were initial hold at 50°C for 2 min and initial denaturation at 95°C for 10 min, followed by 50 cycles consisting of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. The nontemplate control contained water instead of cDNA. Negative cDNA synthesis controls (RNA transcribed without reverse transcriptase) were also employed to reveal possible carry-over contamination. Samples with variation between duplicates larger than 0.5 Cq were reanalyzed. The efficiencies of all three assays were between 90 and 100% and calibration curves had $R^2 > 0.998$. The quantitative real-time PCR study design adhered to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [19].

### Statistical analyses

For statistical analyses, ratios of absolute levels of caspase-2L/*MRPL19*, caspase-2S/*MRPL19* and the caspase-2S/L ratio were calculated. To determine the differences in mRNA expression between tumor and control tissues the nonparametric paired Wilcoxon signed rank test was used. Associations of transcript levels with clinical data were analyzed by nonparametric tests (Kruskal–Wallis, Mann–Whitney and Spearman's rank). The tested clinical data were as follows: age in years; menopausal status (pre- vs post- or peri-menopausal); stage (I or II vs III); tumor size (pT1–2 vs pT3–4); lymph node metastasis (pN0 vs pN1–3); histological type (ductal vs other); grade (G1 vs G2 vs G3); expression of estrogen and progesterone receptors, HER2, Ki-67 and p53 (negative vs positive); and response to the therapy (complete or partial response vs stable disease or progression). Progression-free survival (PFS) was defined as the time elapsed between surgical treatment and disease progression or death from any cause. Kaplan–Meier survival plots with the Log rank test were used for testing PFS as previously published [15]. p-values are departures from two-sided tests. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS v15.0 software (SPSS Inc., IL, USA).

## Results

### Patient characteristics

Comparison of clinical characteristics of both studied sets is presented in Table 1. The neoadjuvant set was composed of patients with a younger age at diagnosis, prevalence of premenopausal status, larger and poorly differentiated (high-grade) tumors and a higher number of patients with lymph nodes affected by metastasis in comparison with the general set. Generally, the neoadjuvant set was composed of patients with considerably more aggressive disease and, thus, worse prognosis than the general set.

### Expression levels of caspase-2 isoforms in breast carcinoma

A significant upregulation of caspase-2L in tumors versus control tissues was observed in paired samples of the general set ($p < 0.001$; $n = 43$; Table 2). However, no significant differences

**Table 1. Clinical characteristics of patients involved in the study.**

| Characteristics | General set; n (%) | Neoadjuvant set; n (%) |
|---|---|---|
| ***Age at diagnosis*** | | |
| Mean age ± standard deviation (years) | 61.4 ± 10.6 | 50.9 ± 10.7 |
| ***Menopausal status*** | | |
| Premenopausal | 10 (10.0) | 30 (46.9) |
| Post-menopausal | 90 (90.0) | 34 (53.1) |
| ***Family anamnesis*** | | |
| Positive | 15 (15.2) | 13 (22.4) |
| Negative | 84 (84.8) | 45 (77.6) |
| Not available | 1 | 6 |
| ***Stage*** | | |
| I | 36 (37.4) | 22 (36.0) |
| II | 41 (42.8) | 32 (52.5) |
| III | 18 (18.8) | 7 (11.5) |
| IV | 1 (1.0) | 0 |
| Not available | 4 | 3 |
| ***Tumor size*** | | |
| Mean size ± standard deviation (mm) | 19.5 ± 11.2 | 23.4 ± 14.8 |
| ***Nodal status*** | | |
| Positive (N1–3) | 42 (43.8) | 27 (42.2) |
| Negative (N0) | 54 (56.2) | 37 (57.8) |
| Not evaluated | 4 | 0 |
| ***Histological type*** | | |
| Invasive duct carcinoma | 83 (83.0) | 54 (84.4) |
| Other type[†] | 17 (17.0) | 10 (15.6) |
| ***Histological grade*** | | |
| 1 | 17 (17.5) | 6 (9.5) |
| 2 | 60 (61.9) | 28 (44.4) |
| 3 | 20 (20.6) | 29 (46.0) |
| Not available | 3 | 1 |
| ***Estrogen receptor status*** | | |
| Positive | 68 (68.0) | 43 (67.2) |
| Negative | 32 (32.0) | 21 (32.8) |
| ***Progesterone receptor status*** | | |
| Positive | 59 (59.0) | 44 (68.8) |
| Negative | 41 (41.0) | 20 (31.2) |
| ***HER2 status*** | | |
| Positive | 28 (28.0) | 16 (25.4) |
| Negative | 72 (72.0) | 47 (74.6) |
| Not available | 0 | 1 |
| ***p53 expression status*** | | |
| Positive | 30 (31.3) | Not evaluated |

[†]*Other tumor types (number in general/neoadjuvant set) involved invasive lobular (9/6), mucinous (3/1), ductal* in situ *(1/0), medullary (1/0), comedo (1/0), papillary (1/0), metaplastic (0/2) and solid neuroendocrine (1/1) carcinomas.*

**Table 1. Clinical characteristics of patients involved in the study (cont.).**

| Characteristics | General set; n (%) | Neoadjuvant set; n (%) |
|---|---|---|
| ***p53 expression status (cont.)*** | | |
| Negative | 66 (68.7) | Not evaluated |
| Not available | 4 | Not evaluated |
| ***Ki-67 expression status*** | | |
| Mean ± standard deviation | Not evaluated | 33.4 ± 23.6 |
| ***Response to neoadjuvant chemotherapy*** | | |
| Complete or partial response | Not applicable | 38 (61.3) |
| Stable disease or progression | Not applicable | 24 (38.7) |
| Not available | Not applicable | 2 |

†*Other tumor types (number in general/neoadjuvant set) involved invasive lobular (9/6), mucinous (3/1), ductal* in situ *(1/0), medullary (1/0), comedo (1/0), papillary (1/0), metaplastic (0/2) and solid neuroendocrine (1/1) carcinomas.*

were found for caspase-2S isoform and the S/L ratio. A weak but significant upregulation of caspase-2L in the tumor compared with the control tissue was also observed in the neoadjuvant set ($p = 0.039$; $n = 38$). The absolute level of caspase-2S and the S/L ratio did not significantly differ between the tumor and control tissue in the neoadjuvant set. The ratios of caspase-2S/L isoforms in tumor and paired control tissues of both studied sets are presented in Figure 1. Relative expression levels of *CASP2L* and *S* in tumors from the neoadjuvant set did not correlate with RIN ($p > 0.05$, $R^2 = 0.049$ for *CASP2L* and $R^2 = 0.034$ for *CASP2S*, respectively) suggesting that RNA quality did not significantly influence the observed results.

### Associations of transcript levels of caspase-2 isoforms with clinical data

Absolute transcript levels of caspase-2L, -2S and S/L ratio in tumor samples were compared with clinical data on patients in both sets separately. A number of associations of both caspase-2L and -2S levels or their ratio with clinical data were observed in both sets of patients. Significant associations observed in at least one set of patients are presented in Table 3–5. For example, transcript level of caspase-2L was significantly higher in patients from the general set with less advanced tumors (stages I or II) compared with stage III tumors (Table 3–5). On the other hand, patients from the neoadjuvant set with lymph nodes affected by metastasis or without expression of estrogen receptors in their tumors had a significantly higher caspase-2L level than node- or receptor-negative patients (Table 3–5). Nevertheless, none of these associations found in the general set were observed in the neoadjuvant set and *vice versa* (Table 3–5). Moreover, patients with a high immunohistochemical protein level of p53 (50% cutoff) had significantly higher caspase-2L levels than patients with a low p53 level in the general set. However, this association could not be evaluated in the neoadjuvant set due to the lack of data on p53 expression. The mean PFS of the general set was 57.1 months. As expected, the established prognostic factors significantly modified the

**Table 2. Differences in the absolute transcript levels of caspase-2L and -2S isoforms between tumor and control tissues from breast carcinoma patients.**

| Tissue type | Caspase-2L/MRPL19 (mean ± standard deviation) | p-value† | Caspase-2S/MRPL19, (mean ± standard deviation‡) | p-value† |
|---|---|---|---|---|
| ***General set*** | | | | |
| Tumors | 0.53 ± 0.20 | <0.001 | 3.88 ± 2.80 | 0.209 |
| Controls | 0.41 ± 0.14 | | 3.18 ± 1.42 | |
| ***Neoadjuvant set*** | | | | |
| Tumors | 1.00 ± 0.38 | 0.039 | 6.50 ± 3.32 | 0.072 |
| Controls | 0.86 ± 0.28 | | 5.49 ± 2.24 | |

†*p-value by two-sided Wilcoxon signed rank test.*
‡*Transcript levels of caspase-2S were multiplied 100-times to demonstrate variation.*

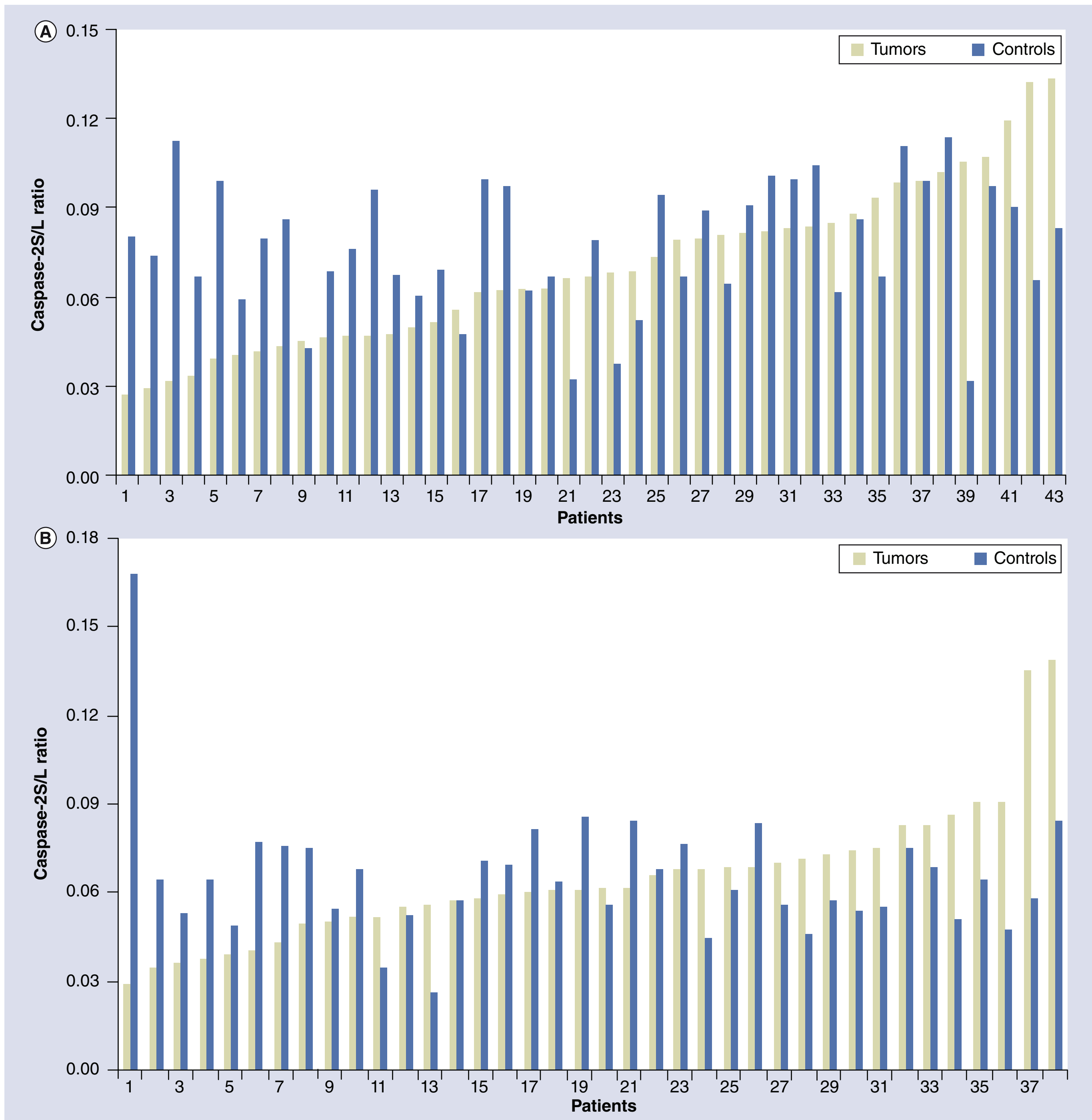


**Figure 1. Caspase-2S/L ratios in tumor and paired control samples from both studied sets.** The **(A)** general (n = 43) and **(B)** neoadjuvant (n = 38) sets of patients.

PFS of patients in the general set. Significantly worse PFS (regardless of the type of therapy) was observed in patients with tumors larger than T2 versus T1 or T2 (p = 0.025), stage III versus I or II (p = 0.040) and negative versus positive expression of progesterone (p = 0.043) and estrogen (p = 0.037) receptors. Menopausal status, tumor type, grade, lymph node metastasis and immunohistochemical expression of HER2 or p53 had no effect on PFS. Transcript levels of caspase-2L or -2S isoforms, or their ratio in tumor tissues of patients in the general set did not significantly modify PFS either in the whole group (n = 88; p = 0.673, 0.955 and 0.348, respectively) or in the subgroup of patients treated by chemotherapy (n = 45; p = 0.912, 0.749 and 0.719, respectively). Kaplan–Meier survival plots for *CASP2L*, *2S* and S/L for the

whole group are shown in Figure 2. Transcript levels of caspase-2 isoforms, or the S/L ratio in tumor tissues did not significantly modify the response of the tumor to neoadjuvant chemotherapy in the neoadjuvant set (p = 0.598, 0.919 and 0.795, respectively).

### Genetic variability of caspase-2 & transcript levels of isoforms

By sequencing caspase-2 regions containing exon 8, alternative exon 9, intronic splicing regulatory element In100, adjacent intronic sequences and exon 10, eight heterozygous carriers of the rs4647321 polymorphism (three in the general set and five in the neoadjuvant set) were identified. No other genetic alterations in blood DNA samples of patients from both sets were found. The observed minor allele frequency for rs4647321 (minor allele frequency: 0.048; n = 83) agreed with the published HapMap Utah residents (Center for the Study of Human Polymorphisms) with northern and western European ancestry sample (minor allele frequency: 0.031, n = 226 [104]; B36 assembly). The presence of the rs4647321 polymorphism did not significantly modify absolute transcript levels of caspase-2L, -2S or S/L ratio.

## Discussion

Poor response to chemotherapy is quite a common phenomenon in anticancer therapy. Numerous reasons are discussed in the literature but molecular markers that would predict the response are generally lacking. Previous *in vitro* studies suggested that caspase-2 may play a key role in apoptosis induced by anticancer drugs [4–6]. However, the majority of current knowledge was obtained using cell and animal models, and there is a limited amount of information about the importance of caspase-2 expression for the prognosis of individual patients and prediction of the chemotherapy efficacy. This study, therefore, aimed at characterization of the role of pro- and antiapoptotic caspase-2L and -2S transcript levels in breast cancer prognosis and therapy outcome.

The general set was composed of samples of tumors and control tissues from unselected breast cancer patients collected prior to any chemotherapy. This set served for exploring the effect of expression of caspase-2 isoforms on prognosis of patients. Additionally, the neoadjuvant set contained patients who underwent neoadjuvant chemotherapy by serial 5-fluorouracil, anthracycline and cyclophosphamide/5-fluorouracil,

**Table 3. Significant associations of transcript levels of caspase-2L/MRPL19 in tumors with clinical data of breast carcinoma patients.**

| Characteristics | General set | | | Neoadjuvant set | | |
|---|---|---|---|---|---|---|
| | *Transcript level (mean ± standard deviation)* | *n* | *p-value*† | *Transcript level (mean ± standard deviation)* | *n* | *p-value*† |
| ***Age at diagnosis*** | | | | | | |
| Age | – | 100‡ | 0.004§ $R^2$ = -0.284 | – | 64‡ | 0.192§ $R^2$ = -0.165 |
| ***Stage*** | | | | | | |
| I or II | 0.53 ± 0.15 | 77 | 0.025 | 0.96 ± 0.35 | 54 | 0.526 |
| III | 0.47 ± 0.25 | 19 | | 0.84 ± 0.03 | 7 | |
| ***Lymph node metastasis*** | | | | | | |
| Positive | 0.51 ± 0.22 | 42 | 0.634 | 1.05 ± 0.40 | 27 | 0.029 |
| Negative | 0.52 ± 0.13 | 54 | | 0.87 ± 0.26 | 37 | |
| ***Estrogen receptor expression*** | | | | | | |
| Positive | 0.50 ± 0.15 | 68 | 0.069 | 0.88 ± 0.30 | 43 | 0.037 |
| Negative | 0.59 ± 0.22 | 32 | | 1.08 ± 0.37 | 21 | |
| ***p53 expression*** | | | | | | |
| Positive | 0.59 ± 0.19 | 30 | 0.030 | Not evaluated | | |
| Negative | 0.50 ± 0.17 | 66 | | Not evaluated | | |

*Only associations significant in either the general or neoadjuvant set of patients are included. Nonsignificant associations with tumor size, histological type and grade, and expression of Ki-67 are not shown.*
*†p-value by two-sided independent Kruskal–Wallis test.*
*‡Number of patients for whom results of correlation between age and expression levels were available.*
*§p-value by Spearman rank correlation.*

**Table 4. Significant associations of transcript levels of caspase-2S/MRPL19 × $10^2$ in tumors with clinical data of breast carcinoma patients.**

| Characteristics | General set | | | Neoadjuvant set | | |
|---|---|---|---|---|---|---|
| | *Transcript level (mean ± standard deviation)* | *n* | *p-value*[†] | *Transcript level (mean ± standard deviation)* | *n* | *p-value*[†] |
| ***Age at diagnosis*** | | | | | | |
| Age | – | 100[‡] | 0.013[§] $R^2$ = -0.248 | – | 64[‡] | 0.072[§] $R^2$ = -0.226 |
| ***Menopause status*** | | | | | | |
| Premenopausal | 5.31 ± 4.28 | 10 | 0.030 | 6.62 ± 3.62 | 30 | 0.223 |
| Postmenopausal | 3.39 ± 1.89 | 90 | | 5.78 ± 3.19 | 34 | |
| ***Lymph node metastasis*** | | | | | | |
| Positive | 3.56 ± 2.88 | 42 | 0.396 | 7.58 ± 4.01 | 27 | 0.012 |
| Negative | 3.50 ± 1.61 | 54 | | 5.14 ± 2.46 | 37 | |
| ***Progesterone receptor expression*** | | | | | | |
| Positive | 3.77 ± 2.42 | 59 | 0.174 | 5.81 ± 3.53 | 44 | 0.049 |
| Negative | 3.30 ± 2.07 | 41 | | 6.97 ± 3.03 | 20 | |
| ***HER2/neu status*** | | | | | | |
| Positive | 3.15 ± 3.03 | 28 | 0.019 | 5.35 ± 3.58 | 16 | 0.134 |
| Negative | 3.75 ± 1.92 | 72 | | 6.49 ± 3.35 | 47 | |

*Only associations significant in either the general or neoadjuvant set of patients are included. Nonsignificant associations with tumor size, histological type and grade, and expression of Ki-67 are not shown.*
[†]*p-value by two-sided independent Kruskal–Wallis test.*
[‡]*Number of patients for whom results of correlation between age and expression levels were available.*
[§]*p-value by Spearman rank correlation.*

epirubicin and cyclophosphamide, and taxane (paclitaxel or docetaxel) regimen, and tumor and control tissues were collected after chemotherapy. The cytofluorimetric and molecular analyses after exposure of DU145 and taxane-resistant DU145-R prostate cancer cells to doxorubicin showed a significant increase in the expression of caspase-2 and -8, CD3 and CD95, before mitochondrial membrane depolarization and caspase-3 activation in both cell lines [20]. In concert with the *in vitro* data discussed above, we aimed to explore the relevance of caspase-2L and -2S isoforms for response to both anthracyclines and taxanes *in vivo* in the neoadjuvant set.

Recently, the proposed tumor suppressor function of caspase-2 was strongly supported [9]. Caspase-2 suppressed lymphomagenesis in response to aberrant c-Myc expression in transgenic mice. Moreover, the accelerated lymphoma onset in caspase-2-lacking mice was associated with reduced rates of p53 loss and increased extranodal dissemination of lymphoma cells, suggesting a role of caspase-2 in cell migration and anoikis [10]. Caspase-2 deficiency also promoted aberrant response to DNA damage and genetic instability in mouse embryonic fibroblasts *in vitro* [21]. Moreover, involvement of caspase-2 in cell cycle checkpoint control, a function clearly linked to cancer development, has also been suggested [22]. However, we only observed a small, although significant, upregulation of caspase-2L transcript in tumor versus control tissue in both sets in our study. Thus, specifically in human breast carcinoma, we cannot confirm the above-suggested tumor suppressor role of caspase-2. Tumor-specific action of caspase-2 in carcinogenesis cannot be ruled out and may present future research directions.

Interestingly, treatment of the human leukemic cell line U937 by various apoptotic stimuli, including paclitaxel, increased the ratio of caspase-2S/L in a time-dependent manner [23]. In addition, topoisomerase I and II inhibitors, such as camptothecin, etoposide or anthracyclines, seemed to drive inclusion of exon 9 to caspase-2S transcript and decrease caspase-2L mRNA and protein [24]. However, *in vivo* studies dealing with expression patterns of both isoforms, especially in breast tumor tissues, were missing until now. In our study, the ratio of caspase-2S/L levels did not significantly differ between tumor and control tissues of both sets. Therefore, it seems that neoadjuvant treatment by taxane- or doxorubicin-based chemotherapy

does not influence the caspase-2S/L ratio *in vivo*.

Caspase-2S was suggested as a candidate for nonsense-mediated decay and low expression of caspase-2S mRNA in comparison with the L isoform was reported *in vitro* [25]. In our experiments, the observed caspase-2L transcript level was six- to 40-times higher than the level of caspase-2S. Despite this fact, the caspase-2S transcript level was unambiguously detected in all studied samples. Caspase-2S mRNA was previously detected in all tumor and non-neoplastic control tissue samples from patients with renal cell carcinoma (n = 36) [26]. Thus, we show that caspase-2S is also expressed in breast carcinomas. Analysis of protein levels by specific antibodies to both L and S isoforms may shed more light on the discrepancy between existing *in vitro* and *in vivo* data. However, the caspase-2S protein product has yet to be conclusively detected. A minor band corresponding in size to the predicted caspase-2S protein sporadically detected by immunoblotting using anticaspase-2 may have been partially processed caspase-2L, which is expected to migrate similarly [27]. Moreover, due to the lack of commercially available antibodies specifically recognizing caspase-2S from -2L it is currently impossible to verify these results on the protein level.

By comparison of the associations of clinical data with caspase-2L, -2S and S/L observed in tumors of general and neoadjuvant sets we did not find any common effect on the prognosis of patients. A significantly higher level of pro-apoptotic caspase-2L was found in tumors from patients with less advanced disease (stage I or II; i.e., in patients with better prognosis compared with stage III patients in the general set). On the contrary, a significantly higher level of caspase-2L in lymph node-positive versus negative and estrogen receptor-negative versus positive status (i.e., with worse prognosis factors) was found in the neoadjuvant set. High levels of the antiapoptotic caspase-2S were associated with the presence of lymph node metastasis and negative status of the progesterone receptor in the neoadjuvant set. However, no such associations were found in the general set. A significantly higher caspase-2S/L ratio was found in patients with versus without lymph node metastasis in the neoadjuvant set and with positive versus negative progesterone receptor status ($p = 0.002$) and negative versus positive HER-2/neu status ($p = 0.004$) in the general set. Most interestingly, levels of caspase-2 isoforms or their ratio did not significantly associate with PFS in the general set or with the response to chemotherapy in the neoadjuvant set. Taken together our results suggest that the role of caspase-2 isoforms in prognosis of breast cancer patients may differ considerably between pre- and post-treatment patients.

Small numbers of patients in the compared groups may be seen as a major limitation of our study and, therefore, results should be interpreted with caution. Associations between caspase-2 isoforms and clinical prognosis factors of breast cancer patients have not been reported this far and so comparison with data of other authors is impossible. A larger study has to be performed to discern the functional consequences of

**Table 5. Significant associations of transcript levels of caspase-2S/L ratio × $10^2$ in tumors with clinical data of breast carcinoma patients.**

| Characteristics | General set | | | Neoadjuvant set | | |
|---|---|---|---|---|---|---|
| | *Transcript level (mean ± standard deviation)* | *n* | *p-value*† | *Transcript level (mean ± standard deviation)* | *n* | *p-value*† |
| ***Lymph node metastasis*** | | | | | | |
| Positive | 6.56 ± 2.96 | 42 | 0.590 | 7.19 ± 2.78 | 27 | 0.029 |
| Negative | 6.75 ± 2.58 | 54 | | 5.79 ± 2.16 | 37 | |
| ***Progesterone receptor expression*** | | | | | | |
| Positive | 7.29 ± 2.72 | 59 | 0.002 | 6.30 ± 2.80 | 44 | 0.202 |
| Negative | 5.74 ± 2.59 | 41 | | 6.56 ± 1.81 | 20 | |
| ***HER2/neu status*** | | | | | | |
| Positive | 5.54 ± 2.52 | 28 | 0.004 | 5.60 ± 1.92 | 16 | 0.249 |
| Negative | 7.09 ± 2.75 | 72 | | 6.67 ± 2.68 | 47 | |

*Only associations significant in either the general or neoadjuvant set of patients are included. Nonsignificant associations with tumor size, histological type and grade, and expression of Ki-67 are not shown.*
*†p-value by two-sided independent Kruskal–Wallis test.*

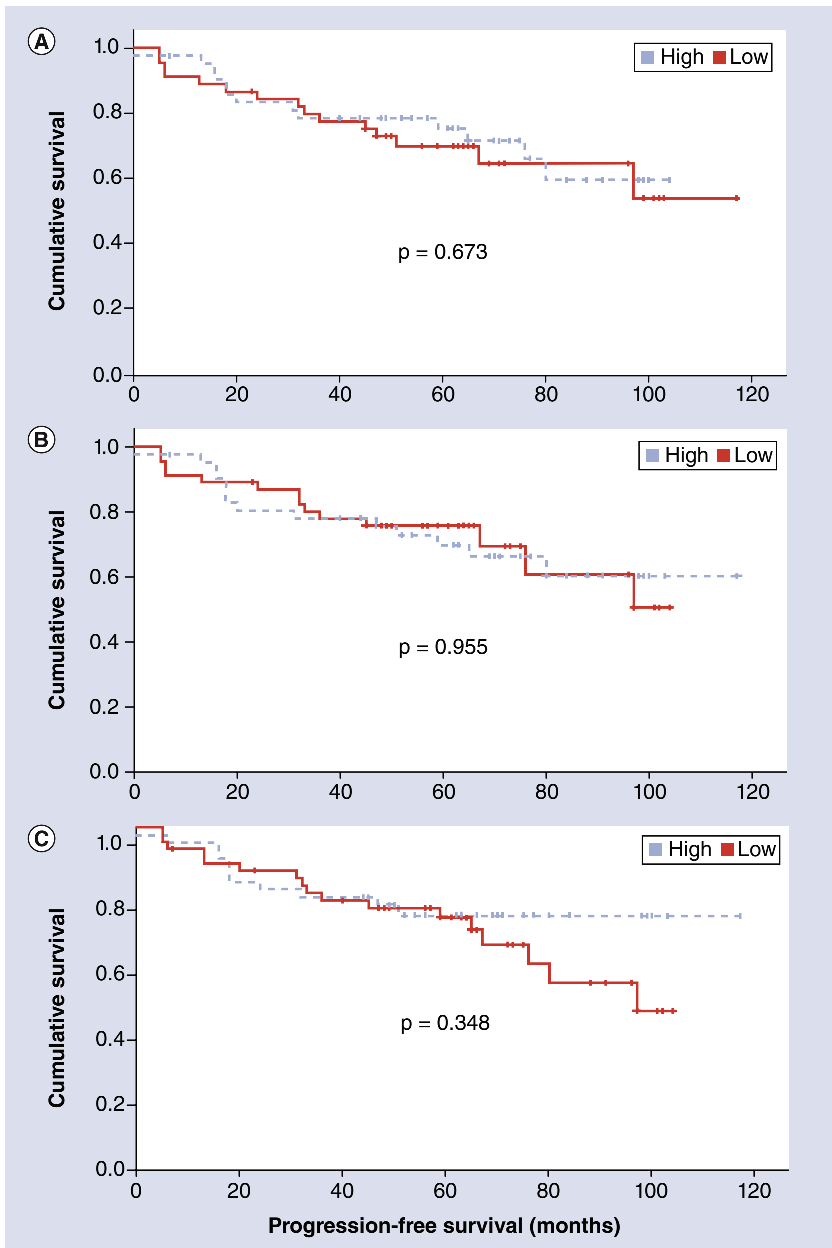


**Figure 2. Kaplan–Meier survival plots for progression-free survival divided by high and low caspase-2L, -2S and S/L expression levels in breast cancer patients. (A)** Caspase-2L, **(B)** -2S and **(C)** S/L levels in tumors of patients from the general set (n = 88) were divided into two categories according to expression levels based on percentiles (high: dotted line; vs low: solid line). Significance of the differences between survival curves was evaluated by the Log rank test.

differences between both sets of patients found here. The lack of both functional data and confirmation of gene expression results by protein analysis also present a notable limitation of our study. Immunoblotting data previously showed a significant ($p < 0.05$) decrease in the expression of caspase-2L, -6, -8, -9 and -3 in breast tumor tissues of different histological grades compared with adjacent uninvolved tissues (n = 10) [28]. These results on a limited sample size seem to contradict our data on caspase-2L transcript level. Thus, a detailed study comparing caspase-2L protein and transcript levels is needed in order to assess the mechanism of regulation of expression and the potential tumor suppressor role of caspase-2L in breast carcinoma.

A growing list of studies using different reagents and cell types indicates a clear role for caspase-2 in stress-mediated and p53-dependent apoptosis [29]. We have observed a significantly higher caspase-2L transcript level in tumors with high p53 expression compared with tumors expressing low p53 levels in the general set. Thus, we may have seen a first example of such association in human tumor specimens.

Introduced mutations of the *CASP2* U/C-rich element upstream of In100 were demonstrated to change the caspase-2S/L transcript ratio [30]. We explored genetic variation of the In100 and its proximity by sequencing DNA from the blood of patients in order to discern eventual impact on caspase-2 alternative splicing. From our results it seems that genetic variability in the In100 region and its proximity does not influence the transcript levels of caspase-2L, -2S and S/L in the followed patients.

## Conclusion

Our study established methods for unambiguous screening of expression of caspase-2 isoforms by absolute quantification. Striking differences in the association of caspase-2 isoforms with clinical prognostic factors between pre- and post-treatment breast carcinoma patients were observed and should be confirmed by follow-up studies. The lack of association with chemotherapy outcome suggests that the previously reported role of caspase-2 isoforms in anticancer drug-induced apoptosis may not be relevant *in vivo*.

## Future perspective

Results of this study do not support the hypothesis that caspase-2 isoforms can be used as universal prognostic or predictive biomarker in breast carcinoma patients. We can not exclude a different prognostic or predictive importance of caspase-2 isoforms in pre- versus post-treatment patients. Thus, a larger follow-up study would be highly valuable in resolving this matter. Differences between transcript and protein levels may exist. Due to the lack of commercially available antibodies specifically recognizing caspase-2S from L we could not address this point. It is, therefore, desirable to produce anti-peptide antibodies against the C-terminus of caspase-2S and to verify our results by protein staining in tumor specimens.

## Executive summary

***Study objectives***

- A key role of caspase-2 in apoptosis induced by anticancer drugs was suggested *in vitro.*
- This study examined transcript levels of caspase-2L and -2S isoforms in breast carcinoma patients and evaluated their prognostic and predictive significance.

***Methodical approach***

- Specific method for absolute quantification of both isoforms was established.

***Major observations***

- Genetic variability of the In100 element and its proximity in blood DNA did not modify levels of caspase-2 isoforms.
- Caspase-2L levels were slightly but significantly higher in tumor versus control tissue in pre- and post-treatment patients, whereas caspase-2S levels did not differ.
- No clear pattern of associations of caspase-2L, -2S or their ratio with clinical prognostic factors was observed in both sets of patients.
- Caspase-2 isoforms or their ratio did not significantly associate with progression-free survival in the pretreatment patients or with chemotherapy response in the post-treatment patients.


#### Financial & competing interests disclosure

*This work was supported by grants from the Czech Science Foundation (no. 301/09/0362) to J Kovář and M Ehrlichová, by the Internal Grant Agency of the Czech Ministry of Health (no.: 13679–4) to P Souček, V Pecha and M Trnková, and by the research project of the Charles University in Prague (PRVOUK P27) to J Kovář. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

*No writing assistance was utilized in the production of this manuscript.*


#### Ethical conduct of research

*The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.*

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