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Klinika dětské hematologie a onkologie

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# Sledování chemorezistence dětských nádorů molekulárně cytogenetickými metodami

*Disertační práce*

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# Obsah

# Abstrakt

Disertační práce se zabývá chemorezistencí dětských nádorových onemocnění a detekcí cytogenetických změn, které s ní souvisí. Ovlivnění rizikových forem dětských nádorů vyžaduje použití vysokých dávek chemoterapie, která v konečném důsledku může vést ke vzniku chemorezistence. Průkaz chromozomálních aberací může přispět k určení prognózy a predikce efektu léčby nádorů i odhalení chemorezistence.

Většinu výsledků jsme získali studiem neuroblastomu. Ostatní prezentované výsledky pak představují genetické změny u Ewingova sarkomu a u dětských feochromocytomů a paragangliomů. Ke studiu chromozomálních aberací byly použity metody komparativní genomová hybridizace (CGH) a array CGH doplněné o vyšetření interfázní fluorescenční hybridizací *in situ* (FISH) případně mnohobarevnou FISH. Změny v expresi na úrovni mRNA byly vyšetřeny expresní čipovou analýzou doplněnou o kvantitativní polymerázovou řetězovou reakci, změny v expresi proteinu byly vyšetřeny pomocí western blottingu nebo průtokovou cytometrií.

Předkládaná disertace je komentovaný soubor šesti publikací. Studium chromozomálních aberací Ewingových sarkomů představuje literární rešerši doplněnou o vlastní výsledky. Práce týkající se feochromocytomů, která je jedním z největších souborů podrobně geneticky vyšetřených dětských feochromocytomů, je zaměřena na hledání chromozomálních aberací. Cílem je přispět k odhalení biologické povahy tohoto nádoru, které není možné na základě histopatologického vyšetření. Nejobsáhlejší část celé práce je založena na sledování cytogenetických změn u neuroblastomu. Dlouhodobou kultivací s rostoucím množstvím ellipticinu jsme připravili linii rezistentní k tomuto cytostatiku. Detekovali jsme cytogenetické změny na úrovni DNA, změny exprese mRNA a vybraných proteinů. Bylo zjištěno, že ABC transportéry se neuplatňují v chemorezistenci k ellipticinu, naopak významná je upregulace topoizomeráz a Bcl-2. Poslední část se týká amplifikace onkogenu *MYCN* - nejdůležitějšího prognostického znaku u neuroblastomu. V této práci jsme detekovali změny v počtu kopií a změny exprese MYCN při *in vitro* léčbě. Jako první jsme prokázali vypuzování amplifikovaných kopií z homogenně se barvících oblastí chromozómů. Navíc jsme zjistili, že po dlouhodobé kultivaci s cytostatikem se zvyšuje exprese MYCN, kterou tak lze považovat za charakteristiku chemorezistentních neuroblastomových buněk. Tyto výsledky mohou být klinicky významné, protože

v terapii neuroblastomu se uvažuje o využití onkogenu *MYCN* jako cíle cílené léčby např. zablokováním genu nebo utlumení jeho exprese RNA interferencí.

Výsledky prezentované v této práci rozšiřují poznatky o genetických změnách a mechanismech chemorezistence u dětských nádorových onemocnění. Práce potvrzuje, že chemorezistence nádorových buněk je podmíněna komplexem mechanismů, které se vzájemně doplňují. Z tohoto důvodu bude k jejich překonání nutný komplexní přístup.

# Abstract

This thesis focuses on drug-resistance in childhood cancer and detection of cytogenetic changes related to it. High-risk forms of childhood cancer often require the use of high doses of chemotherapy, which can ultimately lead to drug-resistance. Detection of chromosomal aberrations of drug-resistant tumors may contribute to the prognosis and prediction of treatment.

Most of the results came from studying neuroblastoma. Other results represent genetic changes in Ewing's sarcoma and pediatric pheochromocytomas and paragangliomas. For the study of chromosomal aberrations we used comparative genomic hybridization (CGH) and array CGH examination supplemented using interphase fluorescence *in situ* hybridization (FISH) or multicolor FISH. Changes in mRNA expression were investigated using expression array analysis complemented by quantitative polymerase chain reactions, changes in protein expression were examined using western blotting or flow cytometry.

This thesis is an annotated collection of six articles. The study of chromosomal aberrations in Ewing sarcomas represents a literature review, together with our results. The study on pheochromocytoma, seeking the biological nature of this tumor (which can not be based on histopathological examination) represents one of the largest sets of chromosomal aberrations in pediatric pheochromocytomas. The most extensive part of the thesis is based on monitoring cytogenetic changes in neuroblastoma. Long-term cultivation, with increasing concentrations of ellipticine, has produced a cell line resistant to this particular cytostatic. We detected cytogenetic changes at the DNA level, as well as changes in mRNA expression and that of selected proteins. We found that ABC transporters did not contribute to ellipticine-resistance, whereas up-regulation of topoisomerases played a significant role as did up-regulation of Bcl-2. The last part of the thesis focuses on the *MYCN* oncogene amplification - the most important prognostic factor relative to neuroblastoma. We detected copy number variations and expression changes in the *MYCN* gene during *in vitro* treatment. We demonstrated the expulsion of amplified *MYCN* copies from the chromosomal homogeneously staining regions. Additionally, we found that long-term cultivation with cytostatic agents increased the expression of MYCN, which can be considered to be characteristic of drug-resistant neuroblastoma cells. These results could be clinically significant

because the *MYCN* oncogene is being considered as a target for neuroblastoma therapy (e.g. blocking or suppression of gene expression by RNA interference).

The results presented in this thesis extend the knowledge regarding genetic changes and mechanisms of drug-resistance in pediatric cancers. The work confirms that drug-resistance of tumor cells is associated with complex mechanisms that complement each other. Therefore overcoming this problem will involve a comprehensive and multifaceted approach.

# Seznam zkratek

| | |
|---|---|
| ABC | **A**TP **B**inding **C**assette |
| aCGH | **a**rray **c**omparative **g**enomic **h**ybridization, čipová srovnávací genomová hybridizace |
| AIDS | **A**cquired **I**mmuno**d**eficiency **S**yndrome, syndrom získaného selhání imunity |
| Amp | **amp**lifikován |
| ATP | **a**denosine **t**ri**p**hosphate, adenosintrifosfát |
| BAC | **b**acterial **a**rtificial **c**hromosome, bakteriální artificiální chromozóm |
| CGH | **c**omparative **g**enomic **h**ybridization, srovnávací genomová hybridizace |
| CNV | **c**opy **n**umber **v**ariation, variabilita v počtu kopií genů |
| DAPI | 4',6-**d**i**a**midino-2-**p**heny**l**indole) |
| DKK3 | **D**ic**kk**opf**3** |
| DNA | **d**eoxyribo**n**ucleic **a**cid**,** deoxyribonukleová kyselina |
| FISH | **f**luorescence ***i**n* **s**itu* **h**ybridization, fluorescenční hybridizace *in situ* |
| GN | **g**anglio**n**euron |
| GNB | **g**anglio**n**euro**b**lastom |
| HIV | **H**uman **i**mmunodeficiency **v**irus, virus lidské imunitní nedostatečnosti |
| HLA | **h**uman **l**eukocyte **a**ntigen, antigen lidských leukoyctů |
| HR NBL | **h**igh-**r**isk **n**euro**bl**astoma, neuroblastomem vysokého rizika |
| HSR | **H**omogenously **S**taining **R**egions, homogenně se barvící oblasti |
| iFISH | **i**nterphase **f**luorescence ***i**n* **s**itu* **h**ybridization, interfázní fluorescenční hybridizace *in situ* |
| INRG | **I**nternational **N**euroblastoma **R**isk **G**roup |
| INSS | **I**nternational **N**euroblastoma **S**taining **S**ystem |
| KDHO | **K**linika **d**ětské **h**ematologie a **o**nkologie UK 2. LF a FN Motol |
| LRP1 | low density **l**ipoprotein **r**eceptor-related **p**rotein **1** |
| MDR1 | **m**ulti**d**rug **r**esistence **p**rotein **1** |
| mBAND | **m**ulticolor chromosome **band**ing, mnohobarevné pruhování chromozómů |
| mFISH | **m**ulticolor **f**luorescence ***i**n* **s**itu* **h**ybridization, mnohobarevná fluorescenční hybridizace *in situ* |

| | |
|---|---|
| MIBG | $I^{131}$**m**eta **i**odo**b**enzyl **g**uanidin |
| mRNA | **m**essenger **R**ibo**n**ucleic **a**cid, informační ribonukleová kyselina |
| MRP1 | **m**ultidrug **r**esistence **p**rotein **1** |
| MYCN | V-**myc** myelocytomatosis viral related oncogene, **n**euroblastoma derived |
| NA | **n**e**a**mplifikován |
| PNET | peripheral **p**rimitive **n**euro**e**ctodermal **t**umor, periferní primitivní neuroektodermální nádor |
| RNA | **R**ibo**n**ucleic **a**cid, ribonukleová kyselina |
| RT PCR | **R**everse **t**ranscription **p**olymerase **c**hain **r**eaction, reverzně transkripční polymerázová řetězová reakce |

# Úvod

Téma své disertační práce Sledování chemorezistence dětských nádorů molekulárně cytogenetickými metodami jsem si vybral na základě zájmu o tuto problematiku daného mým působením v Laboratoři biologie solidních nádorů Kliniky dětské hematologie a onkologie UK, 2. LF a FN Motol, kdy jsem se v rámci vypracovávání diplomové práce seznámil s problematikou studia experimentální chemorezistence a molekulární genetiky nádorů. Téma mě velmi zaujalo a položil jsem si otázkou, které buněčné a molekulární mechanismy chemorezistenci způsobují a v jaké míře jsou podmíněné cytogenetickými změnami nádorové buňky.

# Cíle disertační práce

Cílem disertační práce je studium cytogenetických změn u dětských nádorových onemocnění a s tím spojených expresních změn na úrovni mRNA a proteinu. Snahou je nalézt znaky umožňující stanovení prognózy a/nebo predikci efektu léčby s ohledem na vznik rezistence.

Dílčími cíli bylo:

- Posoudit význam sekundárních chromozomálních aberací u nádorů ze skupiny Ewingova sarkomu.

- U dětských feochromocytomů a paragangliomů metodami molekulární cytogeneticky popsat chromozomální změny, které mohou přispět k určení biologické povahy těchto nádorů.

- U neuroblastomu prokázat molekulární mechanismy chemorezistence k ellipticinu s cílem vyhledat znaky predikující rezistenci k tomuto cytostatiku.

- Zjistit účinky inhibitorů histon deacetyláz (kyseliny valproové a trichostatinu A) na buňky lidského neuroblastomu a přispět tak k objasnění mechanizmů jejich účinků na nádorové buňky a pokusit se vyhledat znaky predikující jejich účinek.

- Studovat změny v počtu kopií onkogenu *MYCN* a jeho expresi v buňkách lidského neuroblastomu po experimentálním navození chemorezistence a při chemoterapii. Význam je v tom, že v současnosti jsou testovány různé formy cílené terapie zaměřené vůči MYCN a tyto poznatky mohou přispět k predikci účinku této terapie.

# 1 Literární úvod

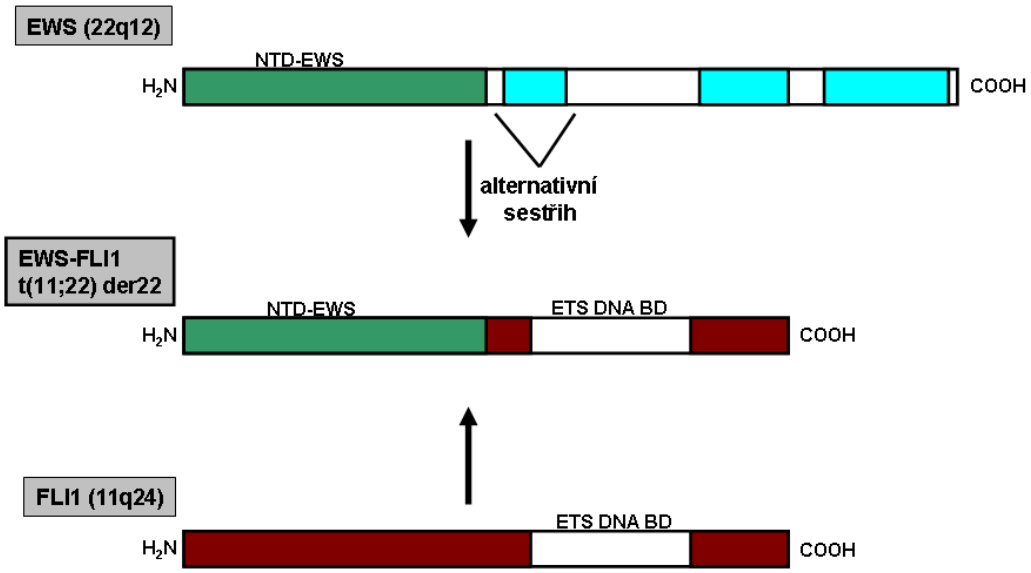
## 1.1 Vybraná nádorová onemocnění dětského věku

Nádory dětí tvoří 1- 3 % všech nádorů. Od nádorů dospělých se liší v epidemiologii, histogenetickém původu i v biologickém chování. Proto je problematika dětských nádorů svébytným medicinským oborem, který je odlišný od onkologie dospělých. Některé nádory se vyskytují jak u dětí tak i u dospělých, mnohdy však mají jiné chování [1].

Typickým nádorem časného dětského věku je neuroblastom se zastoupením přibližně 7-10% nádorů dětského věku, jeho incidence je 8 – 9 na 1 milión dětí mladších patnácti let s průměrným věkem osmnáct měsíců v době stanovení diagnózy [2]. Neuroblastom je maligní embryonální extrakraniální nádor odvozený z nezralých a nediferencovaných buněk neuralní lišty osidlujících paravertebrální sympatická ganglia, dřeň nadledviny a paraganglia. Pro tento nádor je charakteristický velmi variabilní průběh, pohybující se od spontánní regrese nebo vyzrávání v benigní ganglioneurom po vysoce rizikové formy s rozsáhlým onemocněním a často s metastázami, kde přežití dosahuje maximálně 30 %. Neuroblastomové buňky vykazují řadu genetických změn, které se liší u jednotlivých forem a jejichž průkaz přispívá k zařazení do rizikových skupin. Nejčastější jsou ztráta oblasti 1p36 (25 - 35% případů) následované amplifikací onkogenu *MYCN* (asi 20% případů) a ztrátou oblasti 11q23 (15 – 20% případů) [3].

Významnou skupinou nádorů u dětí jsou nádory skupiny Ewingova sarkomu (dále jen Ewingův sarkom). V dnešním pojetí patří do této skupiny vlastní Ewingův sarkom kostí, extraskeletalní Ewingův sarkom a též periferní primitivní neuroektodermalní nádor (peripheral primitive neuroectodermal tumor, PNET) postihující kosti nebo měkké tkáně. Nádory této skupiny jsou po osteosarkomu druhými nejčastějšími primárními maligními nádory kostí starších dětí a mladších dospělých [4]. Ewingův sarkom postihuje nejčastěji pánevní kosti, dlouhé kosti dolních končetin a skelet hrudníku i měkké tkáně v těchto lokalizacích. Na rozdíl od osteosarkomu vychází kostní Ewingův sarkom z diafýzy. Metastazuje do plic, kostí a kostní dřeně, vzácněji do uzlin, jater a mozku. Metastázy jsou přítomny asi u 1/4 pacientů v době stanovení diagnózy. Pro Ewingův sarkom jsou typické chromozomální aberace postihující gen EWS lokalizovaný na 22. chromozómu. Jedná se zejména o translokaci

t(11;22) (q24;q12) spojující gen FLI1 lokalizovaný na 11. chromozómu s genem EWS (Obrázek č. 1). Dále se u Ewingova sarkomu vyskytují translokace t(21;22)(q22;q12) a sporadicky t(7;22)(p22;q12), t(17;22)(q12;q12), t(2;22)(q33;q12) nebo komplexni translokace zahrnujici 11. a 22. chromozom. Produktem těchto translokací jsou chimérické proteiny [5, 6].




**Obrázek č. 1: Schéma translokace EWS-FLI1**. Fúzní gen obsahuje vždy N-terminální transkripční aktivační doménu génu EWS (NTD-EWS) a ETS DNA vazebnou doménu génu FLI1, ale zlomová místa se mohou lišit.

Feochromocytomy se u dětí a dospívajících vyskytují vzácně, přesto jsou nejčastějšími endokrinními nádory dětského věku [7]. Feochromocytomy vycházejí z chromaffiních buněk neurální lišty. Tyto buňky jsou přítomny v dřeni nadledviny. V paraganglionální tkáni nacházející se podél páteře od báze lebky až po pánevní dno vznikají paragangliomy. Tyto dva typy endokrinních nádorů mají velmi podobnou histologii a využívají se u nich stejné terapeutické postupy [8]. Feochromocytomy i paragangliomy jsou charakterizovány zejména syntézou, metabolismem a sekrecí katecholaminů. U těchto nádorů je velmi obtížné určit biologickou povahu na základě histologického obrazu [9]. Většinou jsou maligní formy odhaleny až na základě přítomnosti metastáz. Proto je snahou vyhledat znaky umožňující rozlišit benigní a maligní nádory. Hlavní pozornost je věnována genetickým znakům. Nejčastějšími cytogenetickými změnami u feochromocytomů dětského věku jsou ztráty 1p, části nebo celého 3. chromozómu, 11q, 17p a zmnožení oblasti 9p12 a celého 17. chromozómu [10].

## 1.2 Léčba dětských nádorů

Terapie většiny dětských nádorů se zpravidla zahajuje chirurgickým výkonem. Onkologické operace mohou být radikální, při kterých je celý nádor odstraněn nebo paliativní, které se uplatňují při urgentních stavech a situacích, které zhoršují stav nemocného. Operace diagnostické představující bioptické excize nebo punkce vedoucí k získání tkáně pro histopatologické eventuálně další vyšetření. Pomocné operace jsou prováděny při zavádění centrálního žilního katetru, implantofixu aj. a umožňují další léčebný postup. Rekonstrukční operace jsou prováděny většinou až po ukončení léčby a mají za cíl opravit nebo upravit místo s původní lokalizací nádoru (např. rekonstrukce patra). Preventivní operace předchází chirurgickému odstranění postiženého místa, protože jejím cílem je odstranění potenciálně rizikového místa a pokud možno předejít vývoji nádorového onemocnění. Preventivní operace jsou indikované spíše u dospělých pacientů např. resekce tračníku při familiární polypóze nebo excize kožního ložiska rizikového pro vznik nádoru.

Vzhledem k tomu, že většina nádorů dětského věku je chemosenzitivní po chirurgickém výkonu většinou následuje chemoterapie. Ta se řídí rozsahem, stupněm malignity a přítomností příznivých či nepříznivých prognostických faktorů.

Radioterapie je vyhrazena pouze pro některé nádory, zpravidla pro ošetření zbytku nádoru po operaci a po chemoterapii. Mezi radiosenzitivní nádory můžeme zařadit dysgerminom, embryonální rybdomyosarkom, Ewingův sarkom, hemoblastózy, hemoblastómy, meduloblastom, nefroblastom, neuroblastom a retinoblastom. Naopak mezi radiorezistentní nádory patří osteosarkom, sarkomy měkkých tkání jiné než rabdomyosarkom a teratomy. V pediatrické onkologii je snaha omezit radioterapii pouze na nejnutnější indikace, protože rostoucí zdravé tkáně dětského organizmu jsou radiosenzitivní. Proto se u některých radiosenzitivních nádorů tato léčebná modalita nepoužívá nebo je vyhrazena pouze pro méně příznivé formy jako je to v současné době u Hodgkinova lymfomu, kdy není radioterapie používána při dobré odpovědi na iniciální chemoterapii.

Dětské nádory rostou rychle a časně metastazují, proto je nutné léčit nejen primární nádor, ale i jeho metastázy, což zajistí chemoterapie. Podmínkou úspěšnosti chemoterapie je chemosenzitivita nádoru. Nádory dětského věku se dělí podle této citlivosti na čtyři skupiny (Tabulka č. 1). Účinky protinádorové chemoterapie závisí na velikosti nádoru, intenzitě dávek cytostatik a její toxicitě. Léčiva obtížně pronikají do míst, která jsou nedostatečně prokrvená. Pro tyto oblasti je nutné použít vysokou dávku. Vysokodávkovaná chemoterapie se používá u dětí se špatnou prognózou představující naději na vyléčení pouhých 5-20%. Taková terapie je aplikována na závěr léčby dětí s neuroblastomem vysokého rizika, recidivujícími maligními lymfomy, pokročilými germinálními nádory a Ewingovým sarkomem. Do léčby nejrizikovějších forem Ewingových sarkomů a neuroblastomů je zařazována i megachemoterapie s následnou autologní transplantací hematopoetických progenitorových buněk. V současné době se i u nádorů dětského věku začíná testovat cílená léčba (často nazývaná ne zcela správně „biologická terapie“), která nejčastěji využívá monoklonální protilátky a specifické inhibitory biologicky aktivních molekul, tzv. „malé inhibitory“.

Tabulka č. 1: Rozdělení nádorů dětského věku podle citlivosti k chemoterapii

| Chemoterapie | Příklad nádoru |
|---|---|
| Chemoterapie často vyléčí | leukémie, maligní lymfomy, terminální nádory, nefroblastom |
| Chemoterapie ovlivní pouze částečně | neuroblastom vysokého rizika, osteosarkom |
| Citlivost k chemoterapii je různá | nádory centrálního nervového systému s výjimkou meduloblastomu, maligní melanom a sarkomy nádory měkkých tkání |
| Nádory s nízkou citlivostí | karcinom štítné žlázy, karcinom ledviny, hepatocelulární karcinom |

## 1.3 Vznik chemorezistence a její mechanismy

Chemorezistence je vlastnost buněk, která umožňuje jejich přežití během cytostické léčby. Tuto schopnost mohou mít buňky danou geneticky tj. vrozenou nebo ji získávají po působení cytostatik. Za vyšší citlivostí nádorů na cytostatika stojí obvykle poškození DNA opravných mechanizmů u nádorových buněk [11]. Pokud je cytostatikem zasažena zdravá buňka, je pravděpodobné, že menší poškození opraví. Naopak nádorová buňka má šance na opravu nižší a s větší pravděpodobností podlehne programované buněčné smrti. V chemorezistentních buňkách se mohou v průběhu terapie zefektivnit opravy DNA [12] čímž se chemoterapie stává neúčinnou. Právě individuální schopnost oprav DNA [13] může představovat geneticky danou schopnost chemorezistence. Dalším faktorem, který se uplatňuje u chemosenzitivity je vyšší proliferační frakce v nádorech ve srovnání se zdravými tkáněmi. Většina cytostatik totiž poškozuje převážně proliferující buňky [14].

Nejčastěji uváděným mechanismem chemorezistence je vypuzování cytostatika z buňky pomocí membránových transportérů. Nejznámějšími jsou tzv. ABC (ATP Binding Cassette) transportní proteiny (Tabulky č. 2a a 2b). V literatuře je nejčastěji zmiňovaný ABC transportní protein - P-glykoprotein, který je produktem genu ABCB1

[15]. P-glykoprotein způsobuje rezistenci k antracyklinům, vinka alkaloidům, aktinomycinu D, mitomycinu, 5-fluorouracilu, taxanům i k cílenému léku imatinibu [16]. Schopnost transportovat více léků a způsobit rezistenci k více cytostatikům mu dala pojmenování multidrug resistence protein 1 (MDR1). Druhým mechanismem chemorezistence může být modifikace cílové dráhy nebo cílové struktury pro cytostatikum a také změny nebo mutace v genech či signálních molekulách řídících buněčné dělení a ovlivňujících průběh buněčné smrti. Často se v lékové rezistenci uplatňuje i aktivace drah zabraňujících spuštění programované buněčné smrti nebo naopak deaktivace drah spouštějících apoptózu [17]. Většina těchto změn má genetický základ, který je možný detekovat na úrovni DNA v podobě delecí nebo zmnožení genů. Tyto změny genomu ovlivňují expresi příslušného genu a mění tím vlastnosti buňky. Přehled mechanismů lékové rezistence a genů v nich zahrnutých shrnuje Tabulka č. 3. Odhalení mechanismů chemorezistence a jejich překonání je jedním z klíčových bodů úspěchu chemoterapie nádorů.

Tabulka č. 2a: Chromozomální lokalizace a funkce ABCA a ABCB transportních proteinů.

| Gen | chromozomální lokalizace | funkce proteinu |
|---|---|---|
| *ABCA1* | 9q31 | transport cholesterolu, fosfolipidů a apoptorických tělísek |
| *ABCA2* | 9q34 | **mnohočetná léková rezistence**, transport cholesterolu a fosfolipidů, |
| *ABCA3* | 16p13 | transport lipidů a přispívání k produkci surfaktantů |
| *ABCA4* | 1p22 | transport fosfolipidů specifických pro sítnici |
| *ABCA5* | 17q24 | zatím nejasná funkce v lysozómálním systému |
| *ABCA6* | 17q24 | transport lipidů |
| *ABCA7* | 19p13 | transport cholesterolu a fosfolipidů |
| *ABCA8* | 17q24 | neznámá funkce |
| *ABCA9* | 17q24 | transport lipidů |
| *ABCA10* | 17q24 | transport lipidů |
| *ABCA12* | 2q34 | export lipidů závislých na lamelárních granulích |
| *ABCA13* | 17p312 | neznámá funkce |
| *ABCB1* | 7q21 | **mnohočetná léková rezistence**, záchyt digoxigeninu, úloha v migraci dendritických buněk, regulace střevního vstřebávání, hepatobiliární vylučování, renální sekrece |
| *ABCB2* | 6p21 | transport peptidů do endoplasmatického retikula, význam v expresi HLA I proteinů, sekrece kyseliny žlučové v játrech |
| *ABCB3* | 6p21 | heterodimer s ABCB2 |
| *ABCB4* | 7q21 | **mnohočetná léková rezistence**, transport fosfatidylcholinu |
| *ABCB5* | 7p14 | **léková rezistence**, význam v regulaci buněčné fúze progenitorových buněk |
| *ABCB6* | 2q35 | transport Fe/S složek do cytosolu |
| *ABCB7* | Xq22 | transport Fe/S složek do cytosolu |
| *ABCB8* | 7q36 | transport Fe/S složek do cytosolu |
| *ABCB9* | 12q24 | neznámá funkce |
| *ABCB10* | 1q42 | transport Fe/S složek do cytosolu |
| *ABCB11* | 2q24 | sekrece kyseliny žlučové do jater |

Upraveno podle Hlavata a kol. [18].

Tabulka č. 2b: Chromozomální lokalizace a funkce ABCC, ABCD, ABCE, ABCF a ABCG transportních proteinů.

| Gen | chromozomální lokalizace | funkce proteinu |
|---|---|---|
| *ABCC1* | 16p13 | **mnohočetná léková rezistence**, transport leukotrienů, význam v migraci dendritických buněk |
| *ABCC2* | 10p24 | **mnohočetná léková rezistence**, transport žlučových kyselin a organických aniontů |
| *ABCC3* | 17q21 | **mnohočetná léková rezistence**, transport žlučových kyselin a organických aniontů |
| *ABCC4* | 13q32 | **mnohočetná léková rezistence,** efflux nukleotidů |
| *ABCC5* | 3q27 | **mnohočetná léková rezistence**, efflux nukleotidů |
| *ABCC6* | 16p13 | transport glutathionových konjugátů |
| *ABCC7* | 7q31 | chloridový kanál závislý na protein kináze A |
| *ABCC8* | 11p15 | receptor sulfonylurey |
| *ABCC9* | 12p12 | receptor sulfonylurey, vychytávání glukózy v kosterním svalu |
| *ABCC10* | 6p21 | transport lipofilních anionů, **léková rezistence**, potenciální účast v potlačování NK buněk |
| *ABCC11* | 16q12 | **mnohočetná léková rezistence**, efflux nukleotidů, |
| *ABCC12* | 16q12 | neznámá funkce |
| *ABCC13* | 21q11 | bez aktivity (pseudogen) |
| *ABCD1* | Xq28 | transport dlouhých mastných kyselin v peroxizómech |
| *ABCD2* | 12q11 | transport dlouhých mastných kyselin v peroxizómech |
| *ABCD3* | 1p21 | transport dlouhých mastných kyselin v peroxizómech |
| *ABCD4* | 14q24 | transport dlouhých mastných kyselin v peroxizómech |
| *ABCE1* | 4q31 | formování kapsid viru HIV, inhibice centrální dráhy aktivity interferonu |
| *ABCF1* | 6p21 | zvyšování syntézy proteinů v zánětlivém procesu, aktivace pomocí TNFα |
| *ABCF2* | 7q336 | neznámá funkce |
| *ABCF3* | 3q25 | neznámá funkce |
| *ABCG1* | 21q22 | transport cholesterolu |
| *ABCG2* | 4q22 | **mnohočetná léková rezistence** |
| *ABCG4* | 11q23 | neznámá funkce |
| *ABCG5* | 2p21 | transport sitosterolů |
| *ABCG8* | 2p21 | transport sitosterolů |

Upraveno podle Hlavata a kol. [18].

Tabulka č. 3: Známé mechanismy rezistence k cytostatikům a geny, které se na ní podílí.

| Gen | lokace | protein | funkce | změna při chemorezistenci |
|---|---|---|---|---|
| *ABCB1* | 7q21.12 | P-gp | efflux cytostatika z buňky | overexprese |
| *ABCC1* | 16p13.1 | MRP1 | efflux cytostatika z buňky | overexprese |
| *ABCG2* | 4q22 | BCRP | efflux cytostatika z buňky | overexprese |
| *APEX1* | 14q11.2 | APEX1 | bázová excizní oprava DNA | overexprese |
| *BAX* | 19q13.3-q13.4 | BAX | aktivace apoptózy | downregulace |
| *BCL2* | 18q21.3 | Bcl-2 | blokace apoptózy | overexprese |
| *BRCA1* | 17q21 | BRCA1 | homologní rekombinace | overexprese |
| *BRCA2* | 13q12.3 | BRCA2 | homologní rekombinace | overexprese |
| *CYP1B1* | 1p22.2 | CYP1B1 | metabolismus doxorubicinu | overexprese |
| *ERBB2* | 17q21.1 | c-erb B2/neu | EGFR receptor ovlivňující vnitrobuněčné procesy | overexprese |
| *ERCC1* | 19q13.32 | ERCC1 | nukleotidová excizní oprava | overexprese |
| *ERCC4* | 16p13.12 | XPF | nukleotidová excizní oprava | overexprese |
| *LRP1* | 12q13-q14 | LRP1 | receptor pro přenos signálu a endocytózu | overexprese |
| *MIR34A* | 1p36.22 | - | zvýšená citlivost k apoptóze | downregulace |
| *MIR214* | 1q24.3 | - | deregulace PTEN vedoucí k aktivaci AKT | overexprese |
| *MIR451* | 17q11.2 | - | regulace exprese P-glykoproteinu | downregulace |
| *MLH1* | 3p21.3 | MLH1 | oprava chybného párování bází | metylace |
| *MSH2* | 2p21 | MSH2 | oprava chybného párování bází | metylace |
| *RAD51A* | 15q15.1 | RAD51A | homologií rekombinace a opravy DNA | overexprese |
| *TOP2A* | 17q21-q22 | TOP2A | enzym zodpovědný za topologii DNA | downregulace |
| *TP53* | 17p13.1 | p53 | regulace buněčného cyklu, apoptózy, DNA oprav | mutace |
| *XPA* | 9q22.3 | XPA | nukleotidová excizní oprava | overexprese |
| *XPB* | 2q21 | XPB | nukleotidová excizní oprava | overexprese |

Upraveno podle Rodrigues a kol. [19] a databáze Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/).

## 1.4 Prognóza a predikce

Prognostické klasifikace nádorů jsou založeny zejména na klinických a histopatologických nálezech. Tyto klasifikace však plně neodráží celou šíři biologické rozmanitosti nádorů. I když je určení prognózy nejisté, mají lékaři k dispozici **rizikové faktory** předpovídající možný budoucí průběh choroby. Z hlediska přístupu k pacientovi jsou prognóza a predikce důležitými informacemi. V centru zájmu se ocitají markery, které predikují chování daného nádoru a jeho odpovědi na zvolenou

terapii. Standardní metodou stanovení stupně malignity je rozdělení podle histologické diferenciace tzv. grading. Prognóza onemocnění je stanovována podle lokalizace tumoru, rozsahu postižení a gradu.

Neuroblastom je jedním z prvních nádorů u kterého porozumění klinickým a biologickým faktorům umožnilo volbu léčby a výhled přežití pacientů. Neuroblastom se obvykle šíří do lymfatických uzlin, kostí a kostní dřeně. Nicméně u nejmladších dětí je možné se setkat i s metastázami do jater a kůže (stádium 4S ). Tyto děti mají obvykle velmi dobrou prognózu navzdory přítomnosti metastáz v době stanovení diagnózy (pětileté přežití 92%) [20]. Naproti tomu starší děti mají mnohem horší prognózu a to i pokud nemají v době stanovení diagnózy prokazatelné metastázy [1]. Při volbě optimální léčebné strategie musí být zváženo množství prognostických faktorů zahrnutých do rozhodovacího procesu. Obecně platí, že čím více prognostických faktorů je vybráno, tím lepší rozhodnutí pro léčebnou strategii je možno zvolit. Pro lékaře je vhodné držet se univerzálního protokolu a používat standardní diagnostické a léčebné postupy. Proto byla zahájena spolupráce v rámci the International Neuroblastoma Risk Group (INRG) [15] a byl definován mezinárodní sytém stagingu International Neuroblastoma staining system (INSS) [21]. Výše zmíněná amplifikace onkogenu *MYCN* u neuroblastomu, poprvé popsaná Manfredem Schwabem roku 1984 [22] se stala prvním molekulárně genetickým znakem využitým v dětské onkologii ke stratifikaci léčby [23]. Terapeutický postup pro konkrétního pacienta s neuroblastomem je tak podle současných poznatků založený na kombinaci parametrů: věk pacienta, rozsah onemocnění dle INSS, případná amplifikace *MYCN*, ploidita nádoru a histopatologická klasifikace dle Shimady [24] (Tabulka č. 4). Na základě těchto parametrů pacienty rozdělujeme do rizikových skupin a podle nich provádíme výběr terapie tak, aby vysoce rizikoví pacienti dostali vysoce intenzivní léčbu a na druhé straně nízce rizikoví nebyli zbytečně zatíženi nežádoucími účinky.

Možnost vytipování účinné látky na základě jednoduchého *ex vivo* testu je z více důvodů značně limitovaná a po více než dvou desetiletích testovaní na řadě pracovišť nebyla nikde zavedena jako standard. Jedinou výjimkou je sledování iniciální odpovědi na první fázi léčby (tedy testování chemosenzitivity *in vivo*) u dětí s akutní lymfoblastickou leukémií. Naší snahou je proto nalézt takové znaky v nádorových, ale i zdravých buňkách umožňující předpovídat citlivost k vybraným lékům. Stejně tak nás zajímá výskyt nežádoucích účinků. Prediktivní markery umožňují zvolit co možná

nejefektivnější léčbu nádorových onemocnění za současné minimální zátěže pacienta. Tím se tedy naplňuje alespoň z první části moto Českého národního onkologického programu „správná léčba správnému pacientovi ve správný čas a na správném místě“.

Tabulka č. 4: INRG postup volby terapeutického postupu.

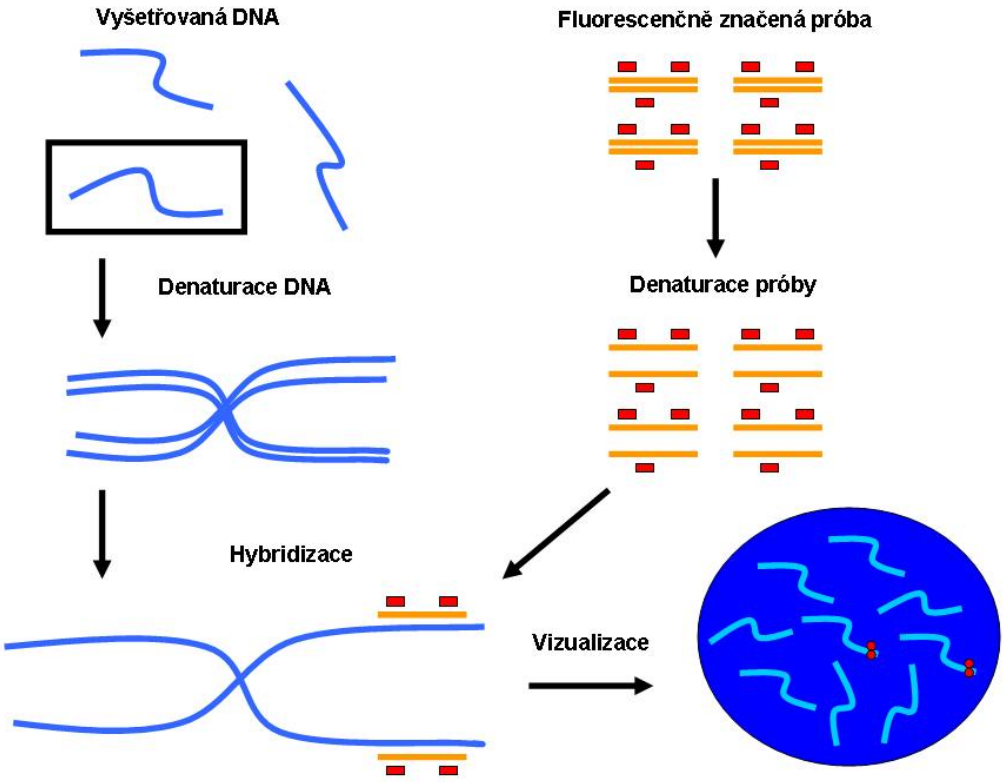
| INRG stádium | věk | histopatologická kategorie | Stupeň diferenciace nádoru | *MYCN* | 11q aberace | ploidie | Terapeutický postup dle rizikové skupiny |
|---|---|---|---|---|---|---|---|
| L1/L2 | | vyzrávající GN; smíšený GNB | | | | | A velmi nízký |
| L1 | | jakýkoli mimo | | NA | | | B velmi nízký |
| | | vyzrávajícího GN nebo smíšeného GNB | | Amp | | | K vysoký |
| L2 | | jakýkoli mimo | | NA | ne | | D nízký |
| | < 18 | vyzrávajícího GN nebo smíšený GNB | | | ano | | K střední |
| | < 18 | nodulární GNB; neuroblastom | diferencovaný | NA | ne | | E nízký |
| | | | | | ano | | H střední |
| | | | slabě diferencovaný nebo nediferencovaný | NA | | | |
| | | | | Amp | | | N vysoký |
| M | < 18 | | | NA | | hyperploidní | F nízký |
| | < 12 | | | NA | | diploidní | I střední |
| | 12 až < 18 | | | NA | | diploidní | J střední |
| | < 18 | | | Amp | | | O vysoký |
| | ≥ 18 | | | | | | P vysoký |
| MS | | | | NA | ne | | C velmi nízký |
| | < 18 | | | | ano | | Q vysoký |
| | | | | Amp | | | R vysoký |

Amp, amplifikován; GN, ganglioneuron; GNB, ganglioneuroblastom; L1, lokalizovaný tumor u kterého nejsou přítomny rizikové faktory detekovatelné zobrazovacími metodami (jsou definovány protokolem např. zavzetí karotid, aorty nebo v. cava do nádoru, komprese trachey nebo hlavních bronchů); L2, lokoregionální tumor; M, vzdálené metastatické onemocnění s výjimkou stádia MS; MS, onemocnění s metastázami omezenými na kůži, játra a/nebo kostní dřeň u dětí do 18 měsíců věku; NA, neamplifikován; Upraveno podle Cohn a kol. [25].

## 1.5 Metody molekulární cytogeneticky

Klinická cytogenetika zjišťuje především strukturní a početní odchylky chromozómů v buňkách s cílem diagnostikovat různé patologické procesy včetně nádorů. Její rozvoj v posledních letech umožnilo zejména zavedení nových způsobů kultivace buněk a barvení chromozómů. Další významný rozvoj umožnila molekulární

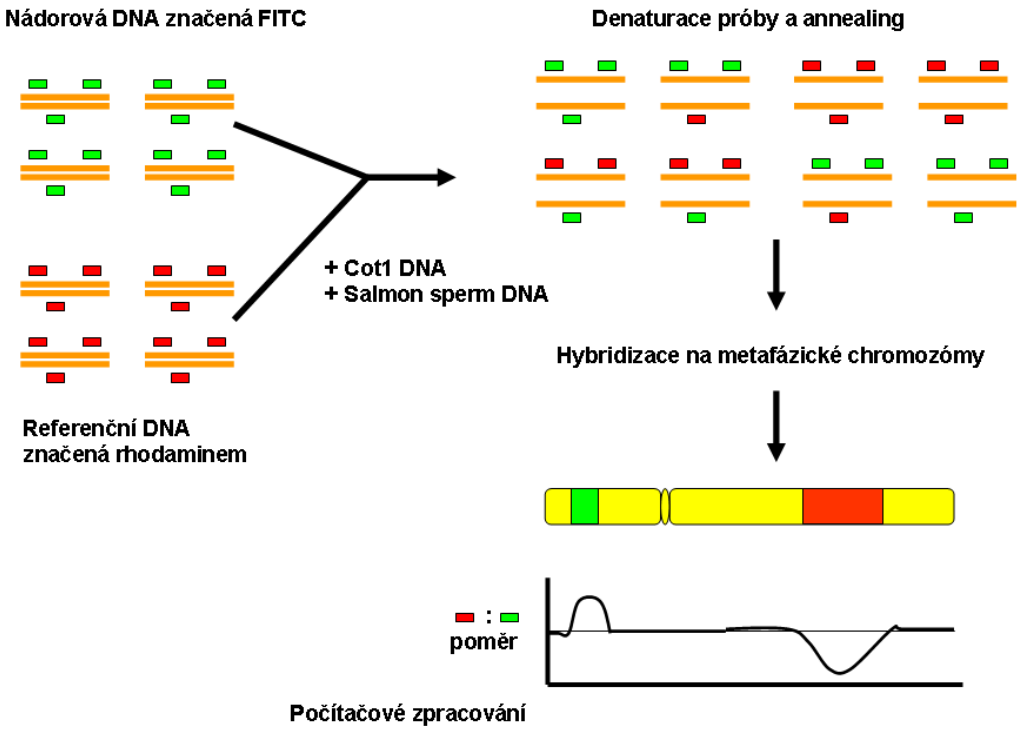
cytogenetika, která studuje geneticky podmíněné změny na molekulární úrovni. Jednou ze základních metod pro studium molekul DNA je fluorescenční hybridizace *in situ* (FISH) (Obrázek č. 2). Metoda vychází ze sedmdesátých let minulého století, kdy byly poprvé použity izotopem značené sondy vysoce repetitivní DNA k průkazu její lokalizace v centromérických oblastech chromozómů myší [26]. Nejdůležitějším momentem pro široké využití metody FISH a jejích dalších modifikací se stal přechod od radioaktivního značení DNA sond k značení neradioaktivnímu - fluorescenčnímu. V průběhu několika posledních let se metody hybridizace *in situ* staly rutinní součástí vyšetření početních a strukturálních změn lidských chromozómů v klinické genetice. Metoda FISH je vhodná pro použití na histologických řezech jak ze zmraženého, tak do parafinu zalitého materiálu, tkáňových preparátech připravených z kultivovaných i nekultivovaných buněk, izolovaných buněčných jádrech a zejména chromozómových preparátech. Sondy používané pro FISH je možné rozdělit na sondy, které hybridizují se specifickými chromozómovými strukturami, což jsou obvykle centromérické oblasti obsahující repetitivní sekvence DNA. Druhou skupinu sond představují genomické klony hybridizující s jedinečnými sekvencemi DNA. Poslední skupinou sond jsou tzv. malovací sondy (chromosome painting probes), které hybridizují s mnohočetnými chromozomálními sekvencemi a s jejich pomocí je možno označit celý chromozóm. [27]. Kombinaci výše uvedených sond můžeme využít k průkazu delecí, translokací, inverzí, zmnožení nebo amplifikací sekvencí DNA. Jednou z nejdůležitější výzkumných aplikací FISH je přímé mapování genů. Při kombinaci tří dostupných fluorochromů (zelený, červený a modrý) můžeme určit i pořadí jednotlivých genů v konkrétních oblastech a zkonstruovat fyzické mapy chromozómů [27].




**Obrázek č. 2: Schéma metody FISH**. Vyšetřovaná dvouvláknová DNA je denaturováná, stejně tak i dvouřetězcová fluorescenčně značená sonda (próba). Denaturovaná próba hybridizuje ke komplementárním úsekům vyšetřované DNA pokud jsou použité vhodné podmínky (teplota, složení hybridizačního pufru). Po obarvení veškeré DNA pomocí barvičky 4',6-diamidino-2-phenylindole (DAPI), která se váže k AT bohatým oblastem DNA, dostáváme ve fluorescenčním mikroskopu modrý signál pro interfázní jádro nebo pro jednotlivé chromozómy v mitóze. Dle použitého fluorescenčního značení próby (v našem případě červené barvičky například rhodaminu) dostáváme odpovídající signál. Pro zdravou buňku v G0 a G1 fázi buněčného cyklu, ve kterých je největší podíl buněčné populace, vyjma pohlavních chromozómů, získáme dva signály.

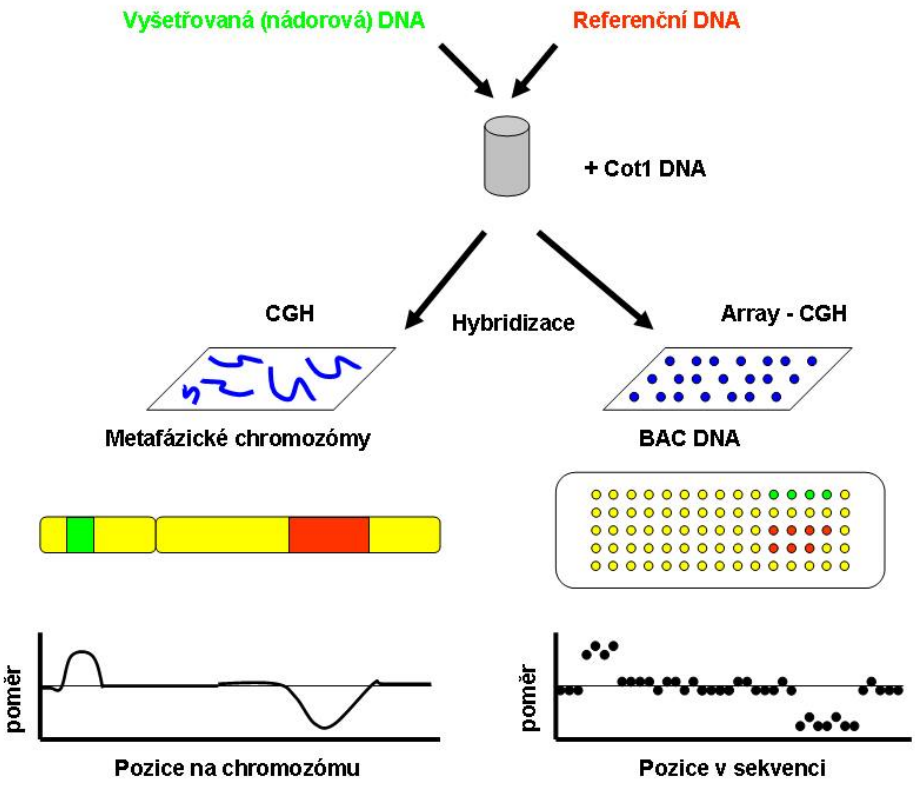
Modifikací hybridizačních technik umožňující v jednom hybridizačním experimentu vyšetřit celý karyotyp včetně pohlavních chromozómů představuje mnohobarevná FISH (multicolor FISH, mFISH). Oproti vyšetření interfázních jader v tzv. interfázní FISH (iFISH) je nutné pro analýzu mFISH získat mitotické buňky a obarvením všech jejich chromozómů lze posoudit všechny přestavby ve vyšetřované buňce. Vzhledem k různým kombinacím flourochromů, které poskytují různé barevné signály nám pro pokrytí celého karyotypu stačí pouze 5 fluorochromů. Citlivost metody se pohybuje v rozmezí 1 – 2,5 Mb. Metodou je možné detekovat kryptické translokace a inzerce, které jsou jinými metodami nezachytitelné. Metoda vycházející z mFISH a která ji vhodně doplňuje a zpřesňuje se nazývá mnohobarevné pruhování chromozómů (multicolor chromosome banding, mBAND) umožňující na vytypovaném chromozómu mnohobarevné pruhování s vysokou rozlišovací schopností [28].

Jednou z možných alternativ FISH v klinické cytogenetice může být metoda srovnávací genomová hybridizace (comparative genomic hybridization, CGH) (Obrázek č. 3) poprvé popsaná před dvaceti lety Kallioniemim a kol. [29]. Metoda využívá současné hybridizace rozdílně fluorescenčně značených DNA, vyšetřované/nádorové s kontrolní DNA. Hybridizace probíhá na metafazické chromozómy odpovídající karyotypu 46,XX nebo 46,XY. Při počítačovém vyhodnocení detekujeme poměr intenzit obou fluorescencí podél všech chromozómů. Při vyšší fluorescenci referenční DNA software vyhodnotí nález jako ztrátu (deleci) a při vyšší fluorescenci vyšetřované DNA jako zmnožení v dané oblasti.




**Obrázek č. 3: Schéma metody CGH**. U klasické CGH jsou kontrolní DNA (referenční DNA, značená červeně) a vyšetřovaná DNA (nádorová DNA, značená zeleně) současně hybridizovány na normální metafázické chromozómy připravené klasickým cytogenetickým způsobem. Po hybridizaci jsou tyto mitózy vizualizovaný a přeneseny pomocí fluorescenčního mikroskopu a černobílé kamery do karyotypovacího softwaru, kde jsou zpracovány. Při CGH vyhodnocení získáváme poměrem intenzit fluorescence jednotlivých chromozomálních oblastí křivku, kterou odpovídající software vyhodnotí jako ztrátu genetického materiálu (převaha referenční DNA) nebo zmnožení (převaha vyšetřované DNA).

Výhodou této metody je vyšetření celého genomu v rámci jednoho vyšetření a možnost využít k analýze DNA izolovanou z nativních nebo zmražených buněk i z materiálu zalitého do parafinu. Nevýhodou je nemožnost pokrytí variability v rámci buněčné populace. Metoda prokáže pouze změny, které jsou přítomny na nejméně ½ buněk (nezachytí tedy změny v minoritním klonu). Druhou nevýhodou je nemožnost detekce balancovaných aberací. Jedná se o metodu, která je časově náročná, protože vyžaduje delší hybridizaci a následné karyotypické zpracování a vyhodnocení. Tato technika je schopna detekovat změny postihující úseky o velikosti nejméně 10 Mb [29]. Další technikou, která je v současnosti používána je array CGH (aCGH), která mapuje genom pomocí hybridizace vyšetřované a kontrolní DNA k jednotlivým známým hybridizačním cílům, které tvoří krátké úseky (bacterial artificial chromosome, BAC) DNA přichycené ke sklíčku. Ve srovnání s CGH tento postup odstranil karyotypování a zvýšil rozlišovací schopnosti metody (Obrázek č. 4). aCGH je široce rozšířená metoda k detekci změn v počtu kopií (copy number variation, CNV), které by mohly mít význam v řadě dědičných i sporadicky se vyskytujících onemocnění včetně nádorových. Například systém NimbleGen CGH microarrays dosahuje při použití 4.2 miliónu sond a průměrné vzdálenosti sond 284 bp vysokého rozlišení až 1.4 kb (www.nimblegen.com).




**Obrázek č. 4: Porovnání CGH a aCGH**. Oproti klasické CGH jsou v případě aCGH kontrolní i vyšetřovaná DNA hybridizovány ke krátkým přesně definovaným úsekům DNA (BAC DNA), které jsou přichyceny (naspotovány) na skleněný podklad čipu. Pozice na čipu jsou přesně určeny a citlivost čipů souvisí s množstvím přichycených úseků DNA na konkrétním čipu. Po hybridizaci jsou čipy skenovány pomocí specializovaných čteček čipů a výsledek je přenesen do počítače. K vyhodnocení čipu je nezbytný software, který příslušnému spotu přiřadí pozici, a tím ho přesně identifikuje a vyhodnotí poměr intenzit fluorescence obou hybridizovaných DNA. Stejně jako u klasické CGH zůstává nemožnost odhalení balancovaných translokací. Výhodou oproti klasické CGH je u aCGH kratší doba analýzy a větší citlivost, kdy při dostatečně velkém množství spotů jsem schopni detekovat změny na úrovni jednotlivých genů.

# 2 Výsledky (Publikace in extenso)

Tato disertační práce se zabývá studiem cytogenetických změn u dětských nádorových onemocnění a s tím spojených expresních změn na úrovni mRNA a proteinu. Smyslem je nalézt znaky umožňující stanovení prognózy a/nebo předpovědět efekt léčby s ohledem na možný vznik rezistence.

Pro studium chromozomálních aberací byl vybrán Ewingův sarkom představující druhý nejčastější primární maligní nádor kostí starších dětí a mladších dospělých. Primární změny a jejich význam pro Ewingův sarkom jsou popsány, ale význam sekundárních chromozomálních aberací Ewingova sarkomu by mohl skrývat diagnostický, prognostický a prediktivní význam. Sumarizace výsledků ze studií zahrnující pacienty s diagnostikovaným Ewingovým sarkomem je předmětem přehledového článku „Nádory ze skupiny Ewingova sarkomu – molekulární biologie a genetika“, kde uvádíme i vlastní dosud nepublikované výsledky. Pro Ewingův sarkom jsou charakteristická častá zmnožení oblasti 1q21-22 a dále trizómie nebo tetrazómie 8. a 12. chromozómu. Pro většinu pacientů je dále typická nebalancovaná translokace t(1; 16) vedoucí ke ztrátě dlouhého raménka 16. chromozómu.

Původní práce „Molecular cytogenetic characterization in four pediatric pheochromocytomas and paragangliomas“, využívající metody molekulární cytogenetiky jako jsou CGH a aCGH, je zaměřena na popis chromozomálních změn u dětských feochromocytomů a paragangliomů. Podobných studií popisujících chromozomální změny u feochromocytomů a paragangliomů je možné nalézt více, ale práce popisující chromozomální změny u dětských feochromocytomů jsou ojedinělé. Hlavní význam spočívá v možnosti nalézt nové genetické markery, které mohou pomoci k určení biologické povahy těchto nádorů. Jedním z těchto nových markerů by mohla být ztráta celého 11. chromozómu nebo pouze jeho krátkého raménka, protože tyto změny jsou častější u feochromocytomů dětského věku než u dospělých pacientů a pravděpodobně mohou mít důležitou úlohu v kancerogenezi dětského feochromocytomu.

Stěžejní práce celé disertace „Mechanisms of ellipticine-mediated resistance in UKF-NB-4 neuroblastoma cells“ zabývající se popisem molekulárních mechanismů chemorezistence k ellipticinu v buňkách lidského neuroblastomu (více viz Tabulka č. 5)

je první zveřejněnou prací zaměřenou na rezistenci k tomuto cytostatiku. Ellipticin je potenciálně použitelný v terapii neuroblastomu. Jsou popsané jeho cytotoxické účinky na buňky neuroblastomu a tato práce významně rozšiřuje znalosti o chemorezistenci neuroblastomových buněk k tomuto cytostatiku. Byly nalezeny mechanismy odpovědné za chemorezistenci k ellipticinu. Jedná se o downregulaci topoizomeráz (ellipticin je kromě jiného inhibitor topoizomeráz), upregulaci Bcl-2 a vypuzování nebo degradaci léčiva. Jako přidružené mechanismy rezistence byly identifikovány upregulace enzymů podílejících se na oxidativní fosforylaci, buněčném dýchání a aerobní respiraci, V-ATPázy, spermine syntetáza, stejně tak pomalejší rychlost růstu rezistentních buněk. Molekulární mechanismy rezistence k ellipticinu byly porovnány s molekulárními mechanismy rezistence k doxorubicinu, cytostatika podobného svými účinky ellipticinu. Zatímco mechanismus účinku léčiv je podobný, mechanismy rezistence k těmto léčivům jsou rozdílné. V rezistenci k ellipticinu nemají význam ABC transportéry zatímco zvýšená exprese P-glykoproteinu je pro rezistenci například k doxorubicinu dominantní. Práce přispívá i k poznání mechanizmů chemorezistence obecně a umožní volbu optimální léčebné strategie.

Tabulka č. 5: Mechanismy rezistence k ellipticinu

| Název genu | Symbol | lokace | mechanismus rezistence |
|---|---|---|---|
| topoizomeráza (DNA) I | *TOP1* | 20q12-q13.1 | downregulace genu kódujícího enzym zodpovědného za topologii DNA |
| topoizomeráza (DNA) II alfa | *TOP2A* | 17q21-q22 | downregulace genu kódujícího enzym zodpovědného za topologii DNA |
| spermin syntetáza | *SMS* | Xp22.1 | upregulace genu kódujícího enzym metabolismu polyaminů |
| spermidine syntáza | *SRM* | 1p36-p22 | upregulace genu kódujícího enzym syntetizující spermidine, který ovlivňuje buněčný růst a diferenciaci |
| B-cell CLL/lymphoma 2 | *BCL2* | 18q21.3 | upregulace genu kódujícího antiapoptotický regulátor |
| RAD23 homolog A (S. cerevisiae) | *RAD23A* | 19p13.2 | upregulace genu kódujícího protein zahrnutý v nukleotidové excizní opravě. |
| xeroderma pigmentosum | *XPA* | 9q22.3 | upregulace genu kódujícího protein zahrnutý v nukleotidové excizní opravě. |
| ATPáza transportující H+ | *ATP6AP1* | Xq28 | upregulace genu kódujícího složku vakuolární ATPázy |
| ATPáza transportující H+ | *ATP6V0D1* | 16q22 | upregulace genu kódujícího složku vakuolární ATPázy |
| ATPáza transportující H+ | *ATP6V0E1* | 5q35.2 | upregulace genu kódujícího složku vakuolární ATPázy |
| ATPáza transportující H+ | ATP6V1F | 7q32 | upregulace genu kódujícího složku vakuolární ATPázy |

V experimentální práci „Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells“ bylo zjištěno, že inhibitory histon deacetyláz v buňkách lidského neuroblastomu ovlivňují expresi cytochromů, které se podílejí na biotransformaci řady léčiv nebo chemických substancí v buňkách. Jedním z těchto léčiv je například ellipticin. Lze předpokládat, že průkaz exprese vybraných cytochromů v nádorových buňkách přispěje k predikci efektu účinku inhibitorů histondeacetyláz.

Jak již bylo zmíněno v literárním úvodu, onkogen *MYCN* je klíčový prognostický marker u neuroblastomu. Studium změn počtu kopií *MYCN* je velmi důležité, protože jeho amplifikace determinuje maligní potenciál buňky. V původním článku „Expulsion of amplified *MYCN* from homogenously staining chromosomal regions in neuroblastoma cell lines after cultivation with cisplatin, doxorubicin, hydroxyurea, and vincristine“ bylo použito několik různých neuroblastomových linií a od nich odvozených linií rezistentních k některým cytostatikům. Tyto linie s amplifikací *MYCN* (IMR-32, UKF-NB-2, UKF-NB-3, UKF-NB-4) i bez amplifikace *MYCN* (SK-N-AS) byly vystaveny účinkům hydroxyurey a dalším cytostatikům užívaným k léčbě neuroblastomu (cisplatině, doxorubicinu a vincristinu). Pomocí cytogenetických metod (CGH a FISH) byl sledován počet kopií *MYCN* v průběhu pasážování buněk. Ukázalo se, že účinkem chemoterapeutik postupně klesá počet kopií genu *MYCN* v senzitivních buňkách a blíží se počtu kopií v buňkách rezistentních k cytostatikům. Například linie UKF-NB-4 ztratila po kultivaci s hydroxyureou průměrně 20 kopií a linie IMR-32 15 kopií ($p < 0.001$). Již po druhé pasáži bylo možné najít buňky bez nadbytečných kopií. Rezistentní buňky ztratily průměrně 17 kopií ve srovnání se senzitivní kontrolou. Tento účinek byl již dříve v literatuře popsán u *MYCN* amplifikovaného ve formě double minutes. Nyní se podařilo poprvé prokázat úbytek kopií též u buněk s amplifikovaným *MYCN* ve formě homogenně se barvící chromozomální oblasti.

Navazující rukopis článku s názvem „Cisplatin impacts the number of *MYCN* gene copies and corresponding expression in human neuroblastoma cell lines“ se zaměřuje na změny exprese onkogenu *MYCN* po navození experimentální chemorezistence a porovnává změny v expresi v závislosti na délce chemoterapie *in vitro* a na počtu kopií *MYCN*. V experimentu byly použity linie s amplifikací *MYCN* (UKF-NB-3, UKF-NB-4) a bez amplifikace (SK-N-AS). Významné je zvýšení počtu

kopií *MYCN* u diploidní linie SK-N-AS po dlouhodobé kultivaci s cisplatinou. Zmnožení kopií *MYCN* u buněk neuroblastomu po léčbě cytostatikem je nalezeno poprvé. S navýšením počtu kopií u této linie souvisí i významně zvýšená exprese mRNA MYCN, což se zdá být charakteristické pro neuroblastomové buňky chemorezistentní k cisplatině. Zatímco v senzitivních buňkách neuroblastomu cisplatina expresi spíše snižuje, v rezistentních buňkách je exprese vysoká bez ohledu na přítomnost nebo nepřítomnost cisplatiny v kultivačním médiu. Význam tohoto článku je v tom, že rozšiřuje poznatky o vztah lékové rezistence k počtu kopií *MYCN* a jeho expresi. Studium vlastností a chování *MYCN* i jeho mRNA a proteinu nabývá na významu, protože v současné době jsou testovány různé formy cílené terapie zaměřené vůči genu *MYCN* i jeho mRNA. Poznatky o změnách v počtu kopií a expresi MYCN mohou přispět k predikci účinku terapie.

## *Seznam prací:*

Práce I:

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Práce IV

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Práce VI

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## Práce I: Nádory ze skupiny Ewingova sarkomu – molekulární biologie a genetika

**Procházka P**, Vícha A, Kodet R, Kodetová D, Eckschlager T. Klin Onkol. 2007; 20(2): 205-8.

**přehled**

# NÁDORY ZE SKUPINY EWINGOVA SARKOMU - MOLEKULÁRNÍ BIOLOGIE A GENETIKA

# EWING SARCOMA FAMILY OF TUMORS - MOLECULAR BIOLOGY AND GENETICS

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**Souhrn**

K nádorům skupiny Ewingova sarkomu (dále jen Ewingův sarkom) patří Ewingův sarkom kostí i extraskeletální a periferní primitivní neuroektodermální nádor. Nádory této skupiny jsou po osteosarkomu druhými nejčastějšími primárními maligními nádory kostí starších dětí a mladých dospělých. Postihují často pánevní kosti, dlouhé kosti dolních končetin a hrudníku a měkké tkáně. Metastazují nejčastěji do plic, kostí a kostní dřeně, vzácněji do uzlin, jater a mozku. Ojediněle byly popsány nádory ze skupiny Ewingova sarkomu vycházející z různých orgánů. Ewingův sarkom se vyznačuje specifickými chromozomálními aberacemi postihujícími 22. chromozom - translokací **t(11;22),** méně často **t(21;22),** sporadicky se mohou vyskytnout i tak zvané minoritní translokace. Translokace specifické pro zařazení do skupiny Ewingova sarkomu spojují část genu EWS lokalizovaného na 22. chromozomu s některým z genů rodiny ETS (FLI-1, ERG, ETV- 1, EIAF, FEV). Kromě těchto specifických translokací nacházíme u Ewingova sarkomu sekundární změny jako jsou trizomie a tetrazomie 8. a 12. chromozomu a nebalancovaná translokace **t(1; 16)**, vedoucí k deleci 16q a ke zmnožení 1q. Význam průkazu těchto sekundárních aberací pro prognózu je předmětem intenzivního výzkumu. Nicméně již nyní se zdá, že translokace t(1;16) a trizomie či tetrazomie 8. chromozomu jsou známkou nepříznivé prognózy.

**Klíčová slova**: Ewingův sarkom, PNET, translokace (11;22) a (21; 22), EWS/FLI1, EWS/ ERG, sekundární chromozomální aberace

**Summary**

Ewing sarcoma of the bones, extraskeletal and peripheral primitive neuroectodermal tumors all belong to the Ewing sarcoma family of tumors. Ewing sarcoma is the second most common primary malignant bone tumor in older children and young adults following osteosarcoma. Pelvic bones, the long bones of lower extremities, as well as the thorax and soft tissue of the above mentioned locations are often affected. They often metastasize into the lungs, bones and bone marrow, but rarely into the nodes, the liver and the brain. Ewing sarcoma was only sporadically reported from different organs. Specific chromosomal aberrations affecting chromosome 22 - translocation **t(11;22),** fewer **t(21;22)**, and the rarely occurring so-called minor translocation - are typical of Ewing sarcoma. Translocations, specific for the Ewing sarcoma family of tumors combine a part of the EWS gene, localized on chromosome 22, with some of the ETS family of genes (FLI-1, ERG, ETV-1 and others). Besides these specific translocations in Ewing sarcoma secondary changes are revealed, such as trisomy and tetrasomy of chromosome 8 and 12 and often nonbalanced translocation t(1; 16) leading to deletion of 16q and amplification of 1q. The significance of establishing these secondary aberrations for prognosis is the focus of intensive research. However, t(1;16) translocation and trisomy or tetrasomy of chromosome 8 are appear to be indications of unfavorable prognosis of the disease.

**Key words**: Ewing sarcoma, PNET, translocation (11;22) and (21; 22), EWS/FLI1, EWS/ ERG, secondary chromosomal aberration.

Buňky v lidském těle jsou vystaveny celé řadě fyzikálních, chemických nebo biologických vlivů, které mohou indukovat poškození jejich genomu. Tyto chromozomální aberace a mutace genů se významně podílejí na vzniku nádorů, přičemž pro vznik a vývoj určitých nádorů jsou specifické konkrétní chromozomální aberace a genové mutace. Z toho vychází molekulárně genetická diagnostika a kombinovaná morfologická a genetická klasifikace nádorů. V dnešním pojetí patří k nádorům skupiny Ewingova sarkomu (dále jen Ewingův sarkom) vlastní Ewingův sarkom kostí, extraskeletální Ewingův sarkom a též periferní primitivní neuroektodermální nádor postihující kosti nebo měkké tkáně (PPNET, zkráceně **PNET**)[1] . Ewingův sarkom postihuje nejčastěji pánevní kosti, dlouhé kosti dolních končetin a hrudníku i měkké tkáně v těchto lokalizacích. Na rozdíl od osteosarkomu vychází kostní Ewingův sarkom z diafyzy. Metastazuje do plic, kostí a kostní dřeně, vzácněji do uzlin, jater a mozku. Metastázy jsou přítomny asi u 1/4 pacientů v době stanovení diagnózy. V literatuře byly popsány kazuistiky Ewingova sarkomu vycházející z různých orgánů například ledviny, močového měchýře, vaječníku, varlete, dělohy, plic, žaludku, slinivky, slinných žláz nebo mesenteria. Na našem pracovišti jsme diagnostikovali PNET ledviny, u kterého jsme prokázali specifickou translokaci t(11; 22) metodou RT PCR nejen v nádoru, ale i v kostní dřeni, což svědčilo pro

minimální metastatické postižení[2]. Ewingův sarkom patří k prognosticky méně příznivým onemocněním dětí a mladistvých a za poslední roky se, na rozdíl od řady jiných nádorů této věkové kategorie, nepodařilo výrazněji zlepšit jeho přežití .

Ewingův sarkom je po osteosarkomu druhým nejčastějším primárním maligním nádorem kostí starších dětí a mladých dospělých. U dospělých nad třicet let a dětí do pěti let je vzácný[3]. Incidence Ewingova sarkomu v populaci mladší dvaceti let je udávána přibližně 2,9 pacientů / milion za rok. Ewingův sarkom se více vyskytuje u mužů, kteří tvoří 55% pacientů s tímto nádorem[4]. Významné rozdíly výskytu jsou patrné v různých populacích. Převažuje postižení bělošské populace. Naopak u asiatů a populace afroamerické i u původních obyvatel subsaharské Afriky je výskyt Ewingova sarkomu šestkrát nižší než u bělochů. Vysvětlením může být délka repetitivních Alu sekvencí intronu 6, nacházejících se blízko oblasti translokačního zlomu Ewingova sarkomu. Alu sekvence jsou retropseudogeny (geny vzniklé zpětnou transkripcí z mRNA transkriptů strukturních genů přemisťovaných transpozicí v rámci genomu ) o velikosti přibližně 300 nukleotidů, tvořící 5% lidské DNA. Repetitivní DNA sekvence, jako například Alu elementy, jsou oblastmi vysoké frekvence zlomů a tedy translokací. Velikost této sekvence v intronu 6 genu EWS je u části osob afrického původu zkrácena na polovinu homologní translokací mezi dvěmi Alu sekvencemi[4]. Na druhé straně z výsledků japonsko - německé studie vyplívá, že prognóza Ewingova sarkomu u bělochů je příznivější než u Japonců[5].

Dlouhou dobu panovaly rozpory v názorech na vznik Ewingova sarkomu. V současné době převládá názor, že nádorové buňky Ewingova sarkomu mají původ v prekurzorových buňkách parasympatiku. Hlavním důkazem je průkaz cholecystokininu v buňkách Ewingova sarkomu a experimentální studie in vitro, kde cAMP, nervový růstový faktor (NGF) nebo forbol-myristát acetát indukovaly v buněčných liniích odvozených od Ewingova sarkomu tvorbu neurofilament[6]. Variabilní lokalizace nádorového onemocnění v kosti nebo měkkých tkáních různé lokalizace je způsobena přítomností pluripotentních kmenových buněk parasympatického nervového systému na mnoha místech organismu. Na původ z neurální tkáně je u Ewingova sarkomu možné usuzovat také z přítomnosti Homer-Wrightových rozet, neurosekretorických granul a imunocytochemicky detekovaných znaků typických pro neurální tkáň[7].

Pro Ewingův sarkom jsou typické chromozomální aberace postihující gen EWS lokalizovaný na 22. chromozomu- **t(11;22)** (q24;q12), méně často **t(21;22)**(q22;q12) a sporadicky **t(7;22)** (p22;q12), **t(17;22)**(q12;q12), **t(2;22)**(q33;q12) nebo komplexní translokace zahrnující 11. a 22. chromozom. Tyto translokace specifické pro zařazení do skupiny Ewingova sarkomu spojují část genu EWS s některým z genů rodiny ETS. Gen FLI-1 je lokalizován na 11. chromozomu, gen ERG na 21. chromozomu, ETV-1 na 7. chromozomu, EIAF na 17. chromozomu a FEV na 2. chromozomu[6,8,9]. U ES byla popsána také inv 22 postihující geny EWS a ZSG[10] Gen EWS se podílí na fúzních genech i u jiných nádorů- EWS/ATF1 (t(12;22)(q13;q12)) u sarkomů z jasných buněk a EWS/WT1 (t(11; 22)(p13;q12)) u desmoplastických nádorů. Méně než 1% nádorů klasifikovaných jako Ewingův sarkom obsahuje fúzní gen FUS/ERG odpovídající translokaci t(16;21)(p11;q22)[11]. U translokace t(11;22)(q24;q12) je podle zlomových míst popisováno více typů. Nejčastější je 1.typ který spojuje exon 7 genu EWS s exonem 6 genu FLI1. 2. typ představuje fúzi exonu 7 genu EWS s exonem 5 genu FLI1. Typ 1 se vyskytuje přibližně u poloviny a typ 2 asi u čtvrtiny nádorů, zbytek tvoří vzácnější typy. Z literárních údajů i našich zkušeností vyplývá, že u nádorů s translokací 2. typu se častěji vyskytují metastázy[12].

Experimentálně byl prokázán význam fúzních genů a jejich proteinů pro maligní zvrat. Transformaci buněk a inhibici apoptózy vyvolá transfekce fibroblastů v tkáňové kultuře fúzním genem EWS/FLI1 - t(11;22)(q24;q12) nebo EWS/ERG - t(21;22)(q22; q12) . Minimální velikost fúzního proteinu postačující k transformaci obsahuje prvních 82 aminokyselin EWS a část proteinu kódované posledním (devátým) exonem genu FLI1 . Více výzkumných týmů již učinilo pokusy o využití poznatků molekulární biologie Ewingova sarkomu v jeho terapii. V experimentech in vitro a in vivo byly testovány antisense nukleotidy a siRNA inhibující fúzní geny EWS/FLI1 nebo EWS/ ERG. Výsledky potvrzují, že inhibice těchto fúzních genů brzdí růst buněk Ewingova sarkomu[14,15].

Kromě uvedených translokací, které jsou považovány za primární, se u Ewingova sarkomu vyskytují také sekundární chromozomální aberace. Nejčastěji je detekována trizomie a tetrazomie 8. a 12. chromozomu a translokace **t(1;16)**(q10-21; q10-13). Tato translokace je často nebalancovaná a vede k deleci 16q a ke zmnožení 1q. Zda se tyto sekundární chromozomální aberace podílejí na vývoji chování Ewingových sarkomů není dosud zcela jasné. Trizomie 8. a 12. chromozomu je podle některých autorů prokazována častěji v nádorech relabujících než v nádorech vyšetřených v době stanovení diagnózy[16,17].

**Vyšetření Ewingova sarkomu metodami molekulární cytogenetiky**

Jedna z prvních studií zaměřená na vyšetřování pacientů postižených Ewingovým sarkomem analyzovala 20 vzorků Ewingových sarkomů od 17 pacientů[18]. Metodou srovnávací genomové hybridizace (CGH)[19-21] bylo identifikováno u 75 % vyšetřovaných nádorů zmnožení a/nebo chybění genetického materiálu. Nejčastější změnou bylo zmnožení celého chromozomálního raménka nebo dokonce celého chromozomu. U 35% nádorů byl zmnožený genetický materiál 8. chromozomu, u 25% zmnožení 1q21-22 a u 25% zmnožení celého 12. chromozomu[18]. Podobné výsledky měla i rozšířená studie této pracovní skupiny. U 75% z 28 vyšetřovaných Ewingových sarkomů byly prokázány genetické změny. Nejčastější bylo zmnožení 8. chromozomu detekované u 36% nádorů. U 5 nádorů byl zmnožený úsek 1q21-22 a 5 nádorů mělo zmnoženou oblast 7q, 11% nádorů mělo zmnožení 6p21.1-pter, zmnožení 12. chromozomu a ztrátu 16q . V jiné studii bylo pomocí CGH vyšetřeno 62 (52 nádorů primárních a 10 recidiv) Ewingových sarkomů. Nejčastější změny zahrnovaly zmnožení 8., 12. a 20. chromozomu a 1q, ztráty 16q a 19q. Byly zjištěny časté kombinace zmnožení 8. a 12. chromozomu, zmnožení 20. chromozomu se zmnožením raménka 8q nebo 18q a ztráta 16q a 17p. Univariantní analýzy ukázaly, že pacienti se zmnožením 1q, 2q a 12. a 20. chromozomu nebo se ztrátou 16q[23] a 17p mají významně nižší celkové přežití než pacienti bez těchto aberací. V multivariantní analýze byla ztráta 16q nezávislým prognostickým faktorem[23]. Brisset a spolupracovníci porovnali 21 lokalizo-

**Tabulka 1.:** Chromozomové změny u Ewingových sarkomů prokázané metodou CGH.
Pozn: Tučně zvýrazněné jsou časté změny. N- počet vyšetřených

| Studie | N | CGH zmnožení | CGH ztráta |
|---|---|---|---|
| Armengol (1997) | **20** | **1q21-22; 8; 12** | |
| Tarkkanen (1999) | **28** | **1q21-22**; 6p21.1-pter; 7q; **8**; **12** | **16q** |
| Ozaki (2001) | **62** | **1q; 8; 12; 20** | **16q; 19p** |
| Brisset (2001) | **43** | **1q; 2**; 5; **8**; **12**; 15; 18; **20** | 16q |

vaných nádorů s 22 metastazujícími nádory. U 63% nádorů (13 lokalizovaných a 14 metastazujících) zjistili genetické změny. Všechny změny se týkaly celých chromozomů nebo celých ramének chromozomů. U lokalizovaných nádorů bylo nejčastější změnou zmnožení 8., 12., 20. a 2. chromozomu. Méně častou změnou bylo zmnožení 5. a 15. chromozomu. V metastazujících nádorech bylo nejčastější změnou zmnožení 8. nebo 12. chromozomu a 1q, méně časté bylo zmnožení 18. a 20. chromozomu. Jedenkrát byla zjištěna ztráta 16q se současným zmnožením 1q představující nebalancovanou translokaci (1;16)[24]. Amiel se spolupracovníky se zaměřil na korelaci změn genomu detekovaných metodami FISH a CGH s telomerázovou aktivitou zjišťovanou pomocí metody TRAPez. V celém souboru prokázali změny genomu u 12 z 15 pacientů. Zvýšená telomerázová aktivita byla prokázána u 10 z 15 testovaných nádorů. 80% nádorů se zvýšenou telomerázovou aktivitou vykazovalo genetické změny. Nebyl tedy rozdíl ve výskytu změn genomu v závislosti na telomerázové aktivitě. Z pacientů s aneuploidními nádory, na rozdíl od dětí s nádory diploidními, nepřežil nikdo 5 let[25]. Tým japonských a německých autorů porovnával chromozomální změny u Ewingova sarkomu detekované metodou CGH u Japonců a bělochů. U Japonců nalezli častěji deleci 19p i 19q než u bělochů. Rovněž byl nalezen rozdíl ve výskytu jednotlivých typů fúzního genu EWS/FLI1- u Evropanů byl méně častý 1.typ .

**Vyšetření Ewingvých sarkomů na KDHO**

Na klinice dětské hematologie a onkologie UK 2.LF a FN Motol jsme v letech 1999-2005 vyšetřili metodou RT PCR[26] 38 vzorků histologicky verifikovaných Ewingových sarkomů. 31x ( 81, 6 %) jsme prokázali fúzní transkript EWS/FL1, 4x (10,5 %) EWS/ERG a 3x (7,9 %) jsme fúzní gen nedetekovali[27]. Naše nálezy jsou ve shodě se zahraničními literárními údaji[8,9].

Ewingovy sarkomy a nediferencované sarkomy morfologicky připomínající Ewingovy sarkomy jsme vyšetřili metodou CGH a RT PCR pro průkaz specifických translokací. U dvou nádorů Ewingova sarkomu hrudní stěny jsme prokázali specifickou translokaci (11;22) a našli zmnožení 8. chromozomu, které je považováno za sekundární změnu provázející horší prognózu. U pacienta s PNETem žebra jsme detekovali specifickou translokaci t(11;22), deleci 16q a zmnožení 1q 9q32-qter, které mohou odpovídat nebalancované translokaci (1;16). Dva ze tří nediferencovaných sarkomů, u kterých nebyla zjištěna translokace t(11;22), měli zmnožení 8. chromozomu. Tato změna tedy není specifická pro Ewingovy sarkomy a je nejspíše sekundární. U našich pacientů s Ewingovými sarkomy jsme nalezli metodou CGH změny popsané v literatuře - zmnožení 8.chromozomu, deleci 16q se zmnožením 1q.

Domníváme se, že přítomnost některých sekundárních změn může být v pozdější době používána k hodnocení prognózy onemocnění a volba léčby tím bude blíže stratifikována. U nádorů skupiny Ewingova sarkomu lze očekávat nalezení molekulárně genetických znaků s prediktivním významem a patrně i uplatnění některé z forem genové terapie (antisense nukleotidy nebo siRNA).

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## Informace - recenze

**AUGUSTÍN PROCHOTSKÝ:**
**KARCINOM HRUBÉHO ČREVA A KONEČNÍKA.**

Litera Medica, Bratislava 2006, náklad 1500 výtisků, ISBN 80-967189-4-0
Monografie o 652 stranách, 1. vydání, předmluvu napsal prof. MUDr. Jiří Hoch, CSc.

Kniha je přehledně členěna do 36 kapitol, ve kterých postupuje autor systematicky od incidence a epidemiologie, etiopatogenezy, rizikových skupin a rizikových faktorů, přes prevenci, screening, symptomatologii, diagnostiku, diferenciální diagnostiku, polypektomie, patologickou anatomii, klasifikaci, progresi a prognostické faktory. Pokračuje úlohou patologa a originálními anatomickými studiemi, různými terapeutickými modalitami včetně léčby lokální, paliativní a adjuvantní. Nepominul pokročilá stadia, léčbu bolesti. Z chirurgických postupů se věnuje hodnocení různých typů anastomóz, technice stomií a rovněž hodnotí přínos laparoskopických přístupů. Dále zmiňuje faktory, ovlivňující výsledky léčby, otázky prevence trombembolické choroby, rizikové faktory a končí dispenzarizací nemocných.
Docent Prochotský dokončil obdivuhodné dílo, které v české i slovenské literatuře v podobném rozsahu dlouho chybělo. Pravděpodobně poslední slovenská monografie na podobné téma banskobystrického Antona Pelikána je z roku 1985. V Česku vyšlo v posledních letech několik stručnějších publikací s tématem kolorektální či rektální karcinom (L. Holubec 2004, Markéta Jablonská 2000, 2004, Kamil Vysloužil 2005).
Na monografii je obdivuhodné, že byla zpracována jediným autorem (kromě kapitoly o léčbě bolesti, na které se podílela Ľ. Nemčíková.
Kniha je formátu A4, velmi kvalitně provedená. Je velice přehledná a navíc obsahuje i CD verzi. Počet citací je rovněž hodný obdivu: (1930). Je bohatě doprovázená schématy i barevnými obrázky (530) a přehlednost zvyšuje i počet tabulek (90).
Publikace je určena nejen začínajícím a zkušeným chirurgům, ale i gastroenterologům a širší lékařské veřejnosti. Mnohá poučení v najdou i patologové a onkologové, jakož i další specialisté.
Kniha má i některé drobné nedostatky. Jako většina slovenských a českých publikací věnuje malou pozornost vlastnímu písemnictví. Stejnou měrou opomíjí publikace našich sousedů a východní Evropy vůbec. Některé kapitoly jsou příliš stručné (kalkulace operačního rizika, karcinom análního kanálu), jiné zase naopak svým rozsahem převyšují význam tématu (mechanická příprava střeva před operací), kam je zařazena navíc i antibioprofylaxe. V kapitole o adjuvantní radioterapii ze tato zaměňuje s terapií neoadjuvantní.
Publikace A. Prochotského by neměla chybět v žádné knihovně chirurgického pracoviště a jistě najde cestu k řadě kolegů s hlubším zájmem o kolorektální karcinom, který je svojí incidencí závažným medicínským a společenským problémem v Česku i na Slovensku.

**SkřičkaT.**

## Práce II: Molecular cytogenetic characterization in four pediatric pheochromocytomas and paragangliomas

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RESEARCH

# Molecular Cytogenetic Characterization in Four Pediatric Pheochromocytomas and Paragangliomas

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**Abstract** Pheochromocytomas (PCCs) are rare tumors among children and adolescents and therefore are not genetically well characterized. The most frequently observed chromosomal changes in PCC are losses of 1p, 3q and/or 3p, 6q, 17p, 11q, 22q, and gains of 9q and 17q. Aberrations involving chromosome 11 are more common in malignant tumors. Unfortunately information about gene aberrations in childhood PCC's is limited. We used comparative genomic hybridization (CGH) and array comparative genomic hybridization (aCGH) to screen for copy number changes in four children suffering from pheochromocytoma or paraganglioma. Patients were diagnosed at the age 13 or 14 years. Bilateral pheochromocytoma was associated with von Hippel-Lindau syndrome (VHL). Multiple paraganglioma was associated with a germline mutation in SDHB. We found very good concordance between the results of CGH and aCGH techniques. Losses were observed more frequently than gains. All cases had a loss of chromosome 11 or 11p. Other aberrations were loss of chromosome 3 and 11 in sporadic pheochromocytoma, and loss of 3p and 11p in pheochromocytoma, which carried the VHL mutation. The deletion of chromosome 1p and other changes were observed in paragangliomas. We conclude that both array CGH and CGH analysis identified similar chromosomal regions involved in tumorigenesis of pheochromocytoma and paragangliomas, but we found 3 discrepancies between the methods. We didn't find any, of the proposed, molecular markers of malignancy in our benign cases and therefore we speculate that molecular cytogenetic examination may be helpful in separating benign and malignant forms in the future.

**Keywords** Pheochromocytoma · Paraganglioma · Comparative genomic hybridization · Pediatric · Microarray

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**Abbreviations**

| | |
|---|---|
| CGH | Comparative genomic hybridization |
| aCGH | Array comparative genomic hybridization |
| PCCS | Pheochromocytomas |
| VHL | Von Hippel-Lindau syndrome |

| | |
|---|---|
| PGL | Paragangliomas |
| NF1 | Neurofibromatosis type 1 gene |
| SDHB, SDHD and SDHC | The genes encoding the succinate dehydrogenase of mitochondrial subunits B, D, and C |
| VMA | Vanillylmandelic acid |

## Introduction

Pheochromocytomas (PCCs) are rare tumors among children and adolescents, despite being the most common pediatric endocrine tumor [1]. They're derived from chromaffin cells that originate from the neural crest. These cells are located in the adrenal medulla (pheochromocytes) and in the paraganglia along the sympathetic chain, and near the aorta. Tumors from extra-adrenal chromaffin tissue are referred to as extra-adrenal pheochromocytomas or paragangliomas (PGL). These two types often share the same clinical course and are histologically equivalent [2, 3]. Twenty percent of all pheochromocytomas occur in childhood [4]. In studies describing pediatric patients, multifocal disease, extra-adrenal disease, and familial association are more frequently described compared to adults. The majority (95%) of pediatric pheochromocytomas are intra-abdominal [5], 40% are bilateral, and 70% are multifocal [4–6]. Although the prevalence of malignancy is commonly cited to be about 10%, other estimates suggest rates of between 3% and 36% of pheochromocytomas/paragangliomas are malignant [7, 8]. Among pediatric patients, approximately 40% of pheochromocytomas are associated with known genetic mutations [9]. At present, the *RET, von Hippel-Lindau gene* (*VHL*), *neurofibromatosis type 1 gene* (*NF1*), *succinate dehydrogenase complex assembly factor 1*(*SDHAF1*), and the genes encoding the B, D, and C subunits of mitochondrial succinate dehydrogenase (*SDHB, SDHD* and *SDHC*) are known to be responsible for tumor formation. The chromosomal locations of these genes are summarized in Table 1. Germline mutations in these genes increase the risk of developing pheochromocytomas and/or paragangliomas, which variably associate with other tumors and characterize different clinical syndromes such as Multiple Endocrine Neoplasia 2 (usually MEN 2A, rarely MEN 2B), von Hippel-Lindau (VHL), and NF 1, or the PGL syndromes. The *SDHB* mutation predisposes patients to extra-adrenal locations and metastatic disease and has been more frequently reported in children [1, 10–12].

Due to the frequency of PCCs in childhood, it has been hypothesized that germline mutations in *RET*, *VHL*, *SDHB*, and *SDHD* cause PCCs more frequently among pediatric patients [9, 13, 14].

**Table 1** Characteristics of genes associated with familial forms of pheochromocytoma

| Gene | Chromosome | Protein |
|---|---|---|
| *VHL* | 3p25-26 | pVHL19 and pVHL30 |
| *SDHB* | 1p36.13 | Catalytic iron-sulfur protein |
| *SDHD* | 11q23 | CybS(membrane-spanning subunit) |
| *SDHC* | 1q21 | CybL (Large subunit) |
| *SDHAF1* | 19q13.12 | Assembly factor 1 |
| *RET* | 10q11.2 | Tyrosine-kinase receptor |
| *NF1* | 17q11.2 | Neurofibromin |

A major problem in PCC relates to the unpredictability of clinical outcomes. Presently there are no defined histological markers to differentiate between benign and malignant PCCs. Features which arouse suspicion of malignancy include large tumor size, small tumor cells, extensive necrosis, vascular invasion, and aneuploidy [15–17]. Only the presence of distant metastases, derived from large pleomorphic chromaffin cells, is widely accepted as a criterion of malignancy [18]. The most frequently observed chromosomal changes in PCC are losses of 1p, 3q and/or 3p, 6q, 17p, 11q, 22q, and gains of 9q and 17q. Aberrations involving chromosome 11 are more common in malignant tumors. Unfortunately, information about gene aberrations in childhood PCC's is limited [19–22].

Applying chromosome comparative genomic hybridization (CGH), we first screened tumor specimens from four pediatric patients to identify genomic aberrations. Next, we validated these findings using array comparative genomic hybridization (aCGH) to increase mapping resolution. This was done because CGH resolution is limited to 10–20 Mb. When we compared results from both techniques, we found some discrepancies. These discrepancies can't be completely explained by limited resolution, indicating that tumor heterogeneity played a role in the discrepancies observed in our results.

## Patients and Methods

A total of four cases were diagnosed and treated in the Department of Pediatric Hematology and Oncology of Motol University Hospital, 2nd Medical Faculty of Charles University, Prague, CZ, between 2003 and 2005.

### Case Histories

#### *Case 1*

An asymptomatic 13-year-old boy was referred to our hospital for hypertension (blood pressure 190/110 mmHg). The

physical examination was entirely normal except for hypertension. A computed tomography (CT) scan examination revealed bilateral adrenal masses (left, 4 cm×3.5 cm×5 cm; right, 5 cm×4 cm×4.5 cm). Biochemical investigation showed an elevated 24-hour urine vanillylmandelic acid (VMA) level of 52.2 mmol/mol of creatinine per day (normal range 0.4–4.0 mmol/mol of creatinine per day). Fundoscopic examination revealed a capillary angioma.

A bilateral resection of the adrenal glands was performed and histological examination of the tissues confirmed pheochromocytoma. Von Hipple-Lindau syndrome was confirmed, DNA sequence analysis revealed a novel germline, heterozygous transversion M_000551:c.374A>C (p.His125Pro) in exon 2 of the *VHL* gene. The mother of the patient was negative for the *VHL* mutation; the father was not examined. The family history was negative for VHL syndrome and PCCs. The patient remains in complete remission (CR) 79 months after diagnosis.

*Case 2*

A 14-year-old boy presented to the emergency department with a history of a single, 2 min, episode of syncope with trismus. He was found, incidentally, to be hypertensive (blood pressure, 160/100 mmHg) with a history of headaches, fatigue, and vomiting. An abdominal CT scan revealed a mass on the right adrenal gland (4 cm× 4.2 cm×4 cm). Biochemical investigation showed an elevated 24-hour urine VMA level of 19.2 mmol/mol of creatinine per day (normal range 0.4–4.0 mmol/mol of creatinine per day). A right adrenal gland resection was performed. Histopathological examination of the tissue confirmed pheochromocytoma.

A germline mutation of the *VHL* gene wasn't identified in this case; however, we found a new somatic heterozygous mutation in the second exon of the *VHL* gene c.389 T>G (p. Val130Gly). We use the Multiplex Ligation-dependent Probe Amplification (MLPA) method to screen for large gene deletions in the VHL and SDH genes. This method was used because large gene deletions account for a considerable proportion of PCC syndromes. A large deletion in the VHL and SDH gene wasn't identified in this case. The family history was negative for VHL syndrome and PCCs. The patient remains in CR 57 months after diagnosis.

*Case 3*

A routine, preventive care, examination by a local pediatrician of a 13-year old boy revealed palpated resistance in the abdomen. A CT scan showed a spherical tumor on the left side of the abdomen (10 cm×8 cm×10 cm) with small local calcifications and hemorrhagic necrosis. Blood pressure was 95/50 mmHg. Twenty-four hour urine VMA was within normal range. A total surgical resection of the tumor was performed. Pathological examination of the tumor tissue confirmed paraganglioma. No germline or somatic mutation of *VHL*, *RET*, *SDHB*, or *SDHD* were found. MLPA was used for detecting large gene deletions in the VHL and SDH genes. While a large deletion in the VHL and SDH genes wasn't identified in peripheral blood, we found loss of one copy of SDHB, and gain of SDHC in the tumor tissue. The patient remains in CR 53 months after diagnosis. The family history was negative for PCCs.

*Case 4*

A 13-year-old girl was examined for a 3 year history of, unilateral (right side), sweating. Over the last 2 years she had suffered from headaches with vomiting two to three times per month. She was found to have hematuria and proteinuria, anisocoria, acute hypertension retinopathy, and hypertension (blood pressure, 223/153 mmHg). The child was referred to pediatric oncology with hypertension and a palpable abdominal mass. CT scan of chest and abdomen revealed a mediastinal mass (4.5 cm×4.5 cm×4.5 cm) and a left retroperitoneal mass (3.5 cm×3 cm×3.5 cm). A total surgical resection of both tumors was performed. The histopathological examination of the two lesions confirmed paraganglioma in both tumors. A germline heterozygous mutation in the *SDHB* gene was identified as *SDHB* 6 c.589 600 dup (p.Cys 196 Cys 200 dup) [23]. The same four-codon duplication was found in her older sister, her father, her paternal uncle, and the uncle's children. All of them were without history of any neoplastic disease. The patient remains in CR 49 months after diagnosis.

## Methods

### Comparative Genomic Hybridization

Tested DNA was extracted from fresh frozen samples to reference DNA came from 20 male to 20 female peripheral blood samples of healthy volunteers. Isolated DNA was mixed together (male or female). DNA was labeled with different fluorochromes using a commercially available kit and carried out according to the manufacturer's instructions (Abbott Molecular, Abbott Park, Illinois, U.S.A.), with a minor modification [24]. Fluorescence imaging and analysis were performed using an Olympus BX51 microscope (Olympus; Tokyo, Japan) and ISIS software (MetaSystems; Altlussheim, Germany). Thirty metaphases were captured and analyzed from each sample. Chromosomal regions were considered to be over-represented if the average green-to-red fluorescence ratio exceeded a cutoff of 1.25

(again) and as under-represented if the ratio was below a cutoff of 0.75 (a loss).

### Array Comparative Genomic Hybridization

We used a commercially available genomic DNA microarray kit (GenoSensor Array 300; Abbott Molecular), which contained DNA representing 287 genes from the BAC, PAC, to P1 libraries. Each cloned DNA was spotted on slides, in triplicate. CGH was performed according to manufacturer's instructions (Abbott-Molecular) and analyzed with a microarray reader and analysis software (GenoSensor Array 300 system, Abbott-Molecular). Spots with G/R ratios more than the mean plus two standard deviations (≈1.2) were considered as gains, while spots with G/R ratios less than the mean minus two standards deviations (0.8) were considered as losses in copy number.

### Gene Analyses

Genomic DNA was extracted from peripheral leukocytes or tumor cells using a salting out method modified according to Miller et al. (1988). We amplified exons 10, 11, and 13 through 16 of the *RET* proto-oncogene according to Neumann et al. (2002). Mutation analysis was carried out using DGGE (Denaturing Gradient Gel Electrophoresis) as previously described [25].

The six exons of the *RET* were amplified in a 25 µl reaction volume with 0.5 µM of each primer, 1x PCR buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP, between 50 and 300 ng of genomic DNA (as a template), and 1 unit of Taq DNA polymerase. PCR was performed for 35 cycles (30 s; 94°C, 45 s; 57–62°C, 40 s; 72°C) with a final extension of 10 min at 72°C. DGGE conditions are available on request. DNA fragments with an aberrant shift on DGGE were analyzed on an automatic fluorescent ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer's instructions.

Exons 1 to 3 of the *VHL* gene and exon-intron boundaries were amplified (primer sequences available on request). PCR was performed in a 30 µl reaction containing 1x PCR buffer, 1.0 mM $MgCl_2$, 100 µM of each dNTP, 0.5 µM of each primer, and 1 unit of Taq DNA polymerase (Fermentas, Lithuania). The PCR conditions were as follows: 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C and extension for 2 min at 72°C, followed by a final extension for 7 min at 72°C. PCR products were then purified using Quick-Clean purification solution (Bioline), and both forward and reverse strands were sequenced using the appropriate PCR-primer and BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on a ABI Prism 3100 Avant Genetic Analyzer (PE BioSystems).

Eight *SDHB* exons and four *SDHD* exons were screened using DGGE. Primers were designed based on GenBank sequences using Primer 3 software (available at: http://www.hgmp.mrc.ac.uk/GenomeWeb/nuc-primer.html) including intron-exon boundaries. The melting profile of DNA fragments, location of primers and GC clamps were analyzed using MacMelt™ software (Bio-Rad, California). The PCR reaction mixture (50 µl) contained 1x PCR buffer (MBI Fermentas), between 50 and 300 ng of genomic DNA (as template), 1.5 mM $MgCl_2$ (MBI Fermentas), 25 pmol of each primer, 200 µM of each deoxynucleotide triphosphate (Promega, USA), and 1.0 units of *Taq*DNA polymerase (MBI Fermentas). The amplification conditions included an initial denaturation at 94°C for 3 min, followed by 35 cycles of 45 s at 94°C, 45 s at 55–65°C (optimal annealing temperature according to the primers conditions), 1 min at 72°C and final extension step running for 5 min at 72°C. DNA fragments exhibiting aberrant band shifts were re-amplified and sequenced in both directions using an automatic fluorescent ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer's instructions.

### MLPA

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used to detect deletions or duplications in the *VHL* or *SDHB, SDHC, SDHD* and *SDHAF1* genes. The SALSA MLPA P016B *VHL* and the P226-B1 SDHD probe kits (MRC-Holland, Amsterdam, Netherlands) were used. The P016B kit contains eight probes to the *VHL* gene (four in exon 1, two in exon 2 and two in exon 3), additional probes to other genes on 3p and control probes to regions telomeric and centromeric from *VHL*. The P226-B1 kit contains nine probes to *SDHB*, seven probes to *SDHC*, five probes to *SDHD* and one probe to *SDHAF1*. Detailed information on probe sequences, gene loci and chromosome locations can be found at www.mlpa.com.

Genomic DNA (50–200 ng) was denatured and the probes were allowed to hybridize (16 h at 60°C). PCR was performed on the samples in a volume of 50 µl containing 10 µl of the ligation reaction mixture and using a thermal cycler Mastercycler ep gradient (Eppendor, Hamburg, Germany). Aliquots of 1 µl of the PCR reaction were combined with 0.5 µl ROX-labelled internal size standard (Applied Biosystems, Foster City, CA, USA) and 12 µl deionized formamide. Fragments were separated by electrophoresis on an Applied 3130XL capillary sequencer and quantified using GeneMarker version 1.6 software (SoftGenetics). For copy-number detection, normal control DNA samples were included in each set of MLPA experiments. Interpretation was based on the comparison

of peak heights between the control DNA and the tumor sample. Cut-off levels for loss of relative copy number were set at 0.75.

## Results

Clinical data and CGH/aCGH results are summarized in Table 2 and Fig. 1. Chromosomal imbalances were observed in all 4 cases. The average amount of genetic aberrations was CGH/aCGH 2.75 and 2.5 changes, respectively (range 2–4) per case. Losses were as common as gains. A comparison of our CGH/aCGH data with data from adult to pediatric patients reported in the literature, together with the Progenetix CGH database (http://www.progenetix.net/progenetix/;14.9. 2009) showed high concordance of the aberration pattern [19–21].

A deletion on chromosome 11 was found in all cases (3×11p, 1×11). Patients with pheochromocytoma showed an aberration pattern distinct from paraganglioma patients. In both pheochromocytomas, there were losses of chromosome 3 or 3p. Paragangliomas showed losses of 1p. In case 4, aCGH detected a 1p deletion, while CGH only detected a partial deletion (1p31.3-ter). Patient 2 had a deletion on chromosome 3 and 11; we didn't find a constitutive *VHL* point mutation, using DNA sequencing, in the DNA extracted from peripheral blood leukocytes. Therefore, we sequenced the DNA from the tumor tissue to detected a new *VHL* mutation, *VHL* c.389 T>G (p. Val130Gly). The patient with the largest tumor, 416 $cm^3$ (case 3), showed the most extensive genetic changes including deletion of 17p11.2-pter and gain of 1q11-qter. In this case, we didn't find a constitutive or somatic mutation. In case 4, we found a discrepancy between CGH and aCGH results on chromosome 17. Therefore, fluorescence in situ hybridization (FISH) was used for validation of these results. We used ON *p53* (17p13)/*MPO* (17q22) ISO 17q" probe (Kreatech Diagnostic) and *RARA* (17q21.1) probes. Testing of nuclei showed diploid status in 45% of *RARA* and 37% of *p53/MPO*. In the majority of nuclei there was aneuploidy, tetraploidy (*RARA* 39%, *p53/MPO* 20.5%), and triploidy (*RAR* 5%, *p53/MPO* 12%). An imbalance was detected in only 25% of nuclei (*MPO/p53*). This is probably due to the heterogeneity of the tumor cell population [26]. Another discrepancy between the techniques utilized in this study was found on chromosome 13, where CGH detected a gain of 13q13-q24, which was at odds with aCGH results. The difference might be explained by a low density of genes, on the chip, at chromosome 13.

We did not observe any correlation between genomic changes and prognosis of the disease. All patients are in the first complete remission following surgical treatment with a median follow up of 55 months.

**Table 2** Clinical and genetic data

| Case | Patient data | | | Tumor characteristic | | CGH/aCGH imbalances | | | MLPA | Follow- up (year) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sex/age (year) | Germinal mutation | somatic mutation | size ($cm^3$) | Origin | | Loss | Gain | | |
| 1 | m/13 | Het.-*VHL* c.374A>C | n.d | 36+47 | pheochromocytoma | CGH | 3p12-pter;11p11-pter | – | n.d | CR (6.58) |
| | | | | | | aCGH | 3p14.2-p26; 11p15.5-pter | – | | |
| 2 | m/14 | neg. | Het.-VHL c.389 T>G | 35 | pheochromocytoma | CGH | 3;11 | – | GGA. negative | CR (4.75) |
| | | | | | | aCGH | 3;11 | – | | |
| 3 | m/13 | neg. | neg. | 416 | paraganglioma | CGH | 1p11-pter;11p11-pter;17p12-pter | 1q11-qter | GGA negative | CR (4.41) |
| | | | | | | aCGH | 1p13.1-pter;11p13-pter; 17p11.2-pter | 1q11-qter | SGA del SDHB; gain SDHC | |
| 4 | f/13 | Het.- *SDHB* 6 c.589-600dup. AGC ACC AGC TGC | n.d | 47+19 | paraganglioma | CGH | 1p31.3-pter; 11p15.1-pter;17p11.2-ter[a] | 13q13-q24 | n.d | CR (4.00) |
| | | | | | | aCGH | 1p12-pter; 11p13-pter | 17p[a] | | |

*n.d.* not done, *neg.* negative, *GGA* germinal gene alteration, *SGA.* somatic gene alteration, *CR* complete remission, *m* male, *f* female [a] FISH found heterogeneity in status of 17p, *Het.* heterozygous

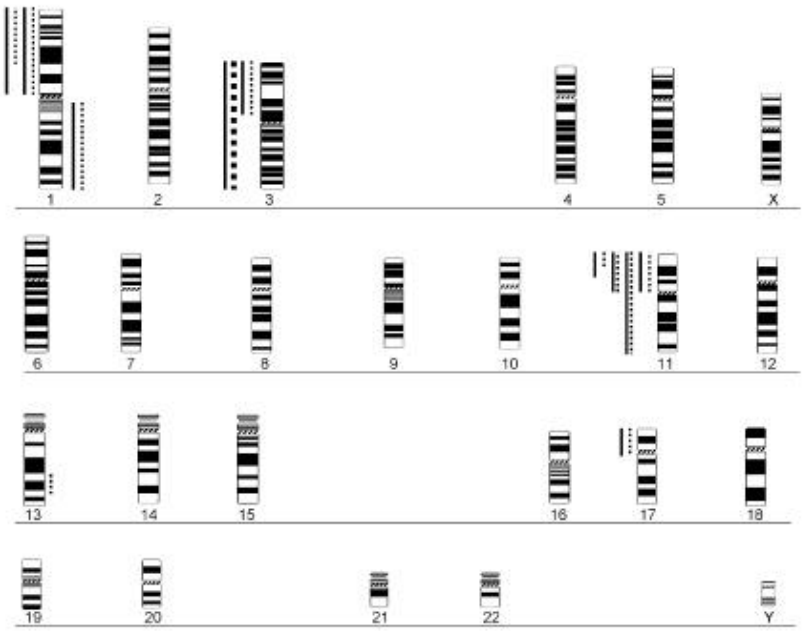


**Fig. 1** Frequency plot of genetic changes for all 4 PCC. Loss and gain of chromosomal material are depicted by vertical bars to the left (loss) and right (gain) of chromosomes, respectively. *Dashed lines* indicate chromosomal CGH and normal lines aCGH. A deletion on chromosome 11 was found in all cases (3×11p, 1×11). Patients with pheochromocytoma showed an aberration pattern distinct from paraganglioma patients. In both pheochromocytomas, there were losses of chromosome 3 or 3p. Paragangliomas showed losses of 1p

## Discussion

In agreement with other studies [4, 19–22], we found unbalanced chromosomal aberrations in PCCs, using CGH. This suggests that chromosomal changes might be an important tumorigenic event. Data from the literature shows that the most common copy number changes in PCC include loss of chromosomes 1p, 3q, 3p, 11p,11q, 6q, 17p, 22 and gain of chromosome 9q, 17q, and 20q [19–22, 27]. In our pediatric study, the most commonly observed chromosomal imbalances in PCCs included 1p, 3p, and 11p. All cases had more than one unbalanced change. These findings support the hypothesis of Koshla et al. [28] regarding involvement of multiple genes in the pathogenesis of these tumors.

Lui et al. [22], in a study of adult patients with PCC, reported a strong association between *VHL* mutations and loss of chromosomes 3 and 11. Hering's and our data suggest that mutations in *VHL*, which are either hereditary or somatic in origin, are also associated with 3p and 11p deletions. Hering et al. [21] identified a combined deletion of 3p and 11p in only 40% and combined deletion 3 and 11 in the remaining cases of VHL-associated PCC. In our study, we found loss of chromosome 11 or 11p in all cases (*VHL*-related pheochromocytoma and also in paraganglioma). Dannenberg et al. [19] detected the loss of 11p in two out of nine sporadic paragangliomas using CGH. Furthermore, loss of 11p has been reported in 5 of 11 sporadic abdominal paragangliomas [20]. We also detected deletion of 11p in a case of abdominal paraganglioma involving a mutation of the SDHB gene.

Numerous cases of deletion of chromosome 11 or 11p support the hypothesis that genes, relevant to PCC, are on the p arm of chromosome 11. Potential candidate genes are numerous and include WT1, IGF2, BW1, CDKN1C, H19 and others. Imprinting effects are important in some of these genes [21, 29].

Malignant pheochromocytomas represent very rare childhood tumors. Older age, absence of genetic syndromes in the family history, and DNA diploid tumors are favorable, relative to outcomes in pediatric PCC. The distinction between benign and malignant PCC cannot be made on the basis of clinical, biochemical, or histopathologic characteristics [4]. Data on genetic events, which could determine the malignant potential of PCCs are, so far, unsatisfactory, but some chromosomal changes (deletion 11q22-qter, deletion 6q) and aneuploidy are found more often in malignant tumors.

Edström et al. [20] showed that the main difference between benign and malignant tumors was partial deletion or gain of chromosome 11, as observed in 9 out of 12 malignant cases and 3 of 16 benign tumors. Among nine patients which developed metastasis, eight showed involvement of chromosome 11. Loss of 11q22-23 was significantly more common in malignant tumors than in benign ones [20]. Deletion of 11q22-23 has been described, by Hering, in patients with metastatic disease, which might strongly suggest the malignant potential of PCCs [21].

None of the tumors in our study showed loss of 11q22-qter as a solo aberration.

Frequent allelic imbalances at 6q have been reported in other malignancies and appear to be related to a poor prognosis or metastatic disease [30–32]. Dennenberg et al. [19] detected a loss of 6q in 34% of sporadic pheochromocytomas. These deletions were strongly associated with metastatic disease, although, Lemeta et al. [3] found that 72% of pheochromocytomas, including tumors classified as either benign or borderline, showed allele loss at 6q in two commonly affected regions (6q14 and 6q23-24). All cases were sporadic PCCs and the authors didn't find any significant difference in the allele loss between benign and borderline tumors. August et al. [33] was unable to confirm that a loss of 6q was an important event in tumor progression. CGH and aCGH did not revealed chromosomal changes on chromosome 6 in our cases.

Gain of genetic material is more frequently associated with malignant courses. The total number of genetic aberrations is higher in malignant tumors compared to benign tumors. Edström et al. [20] found a wide range in a number of genetic aberrations in both malignant tumors (mean = 6) and benign tumors (mean = 2.5). Dannenberg et al. [19] observed only a marginal association between the mean number of chromosomal alterations and malignancy (5.3±2.7 versus 8.2±6.1). August et al. [33] showed that tumors with 10 or more copy number changes were always associated with the development of metastases at a later stage, the presence of 8 chromosomal aberrations was associated with the occurrence of metastases in 85% of cases, while 60% of metastatic tumors showed less than 6 chromosomal aberrations. In our study the average of genetic aberrations, as revealed by CGH/aCGH, was 2.75 and 2.5 (range 2–4) per case, respectively. Additionally, we didn't find any of the 'supposed' molecular markers of malignancy in our patients.

In conclusion, our results showed which copy number changes, were the most common copy number changes in PCC's. Regarding the most common changes (1p, 3, 3p, 11, 11p), both techniques yielded similar results, however, we found 3 discrepancies between the methods. These discrepancies can't be completely explained by limited resolution, indicating that tumor heterogeneity played a role in the discrepancies observed in our results.

Our observations lead us to suggest that the incidence of deletion of chromosome 11 or 11p is more common in childhood PCC, than in adult PCC. These copy number alterations may play a significant role in PPC tumorigenesis.

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## Práce III: Mechanisms of ellipticine-mediated resistance in UKF-NB-4 neuroblastoma cells

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# Mechanisms of ellipticine-mediated resistance in UKF-NB-4 neuroblastoma cells

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**Most high-risk neuroblastomas develop resistance to cytostatics and therefore there is a need to develop new drugs. In previous studies, we found that ellipticine induces apoptosis in human neuroblastoma cells. We also investigated whether ellipticine was able to induce resistance in the UKF-NB-4 neuroblastoma line and concluded that it may be possible after long-term treatment with increasing concentrations of ellipticine. The aim of the present study was to investigate the mechanisms responsible for ellipticine resistance. To elucidate the mechanisms involved, we used the ellipticine-resistant subline UKF-NB-4$^{ELLI}$ and performed comparative genomic hybridization, multicolor and interphase FISH, expression microarray, real-time RT-PCR, flow cytometry and western blotting analysis of proteins. On the basis of our results, it appears that ellipticine resistance in neuroblastoma is caused by a combination of overexpression of Bcl-2, efflux or degradation of the drug and downregulation of topoisomerases. Other mechanisms, such as upregulation of enzymes involved in oxidative phosphorylation, cellular respiration, V-ATPases, aerobic respiration or spermine synthetase, as well as reduced growth rate, may also be involved. Some changes are expressed at the DNA level, including gains, amplifications or deletions. The present study demonstrates that resistance to ellipticine is caused by a combination of mechanisms. (*Cancer Sci* 2012; 103: 334–341)**

Neuroblastoma (NBL) is the most common extracranial solid tumor in children. Neuroblastomas are biologically heterogeneous and differ in their genetic characteristics. Prognostic groups are characterized by genomic changes. Approximately 40% of NBL are in the high-risk NBL group (HR-NBL), which is characterized by structural changes.[1] Improvements in the treatment of HR-NBL in the past decade have not substantially improved prognosis compared with other pediatric malignancies.[2] Chemotherapy is ultimately ineffective in curing HR-NBL because drug resistance arises in most patients, despite intensive therapy.[3,4] Recent studies have provided a link between increased metastatic potential and drug-resistant phenotypes, indicating that, in addition to the development of drug resistance, chemotherapy may cause changes in the biological characteristics of tumors.[5] Treatment of older children with disseminated NBL continues to be a considerable challenge for pediatric oncologists. The drugs used in the treatment of NBL include platinum compounds, alkylating agents, topoisomerase II (TOP2) inhibitors, anthracyclines and vincristine.[1] Recently, we suggested a novel treatment for NBL using the alkaloid ellipticine. We found that exposure of human NBL cell lines, resulted in the induction of apoptosis.[6,7] These effects were associated with the formation of covalent ellipticine-derived DNA adducts formed by cytochrome P450 (CYP)- and peroxidase-derived ellipticine metabolites. In addition to DNA adducts, the toxicity of ellipticine against NBL also involves the intercalation of DNA[8,9] and inhibition of TOP2A.[9–11] Because drug resistance is a feature of HR-NBL, the mechanisms involved in the development of resistance by NBL to anticancer drugs has been investigated.[12–14] However, there is little information available regarding the resistance to ellipticine in NBL. Previously, we determined that ellipticine was able to induce resistance in the NBL cell line UKF-NB-4; specifically, we found that ellipticine may induce resistance in UKF-NB-4 cells after long-term treatment with increasing concentrations.[6]

The aim of the present study was to investigate the mechanisms responsible for ellipticine-induced resistance in UKF-NB-4 cells. This was determined by analyzing the genetic programs in these cells. Several approaches were used, including molecular cytogenetic (comparative genomic hybridization [CGH]), multicolor (m) and interphase (i) FISH, mRNA expression detection (microarray and real-time quantitative [q] RT-PCR), flow cytometry, western blotting and membrane lipid packing detection.

## Materials and Methods

**Cell lines.** The UKF-NB-4 cell line that was established from recurrent bone marrow metastases of HR-NBL[15] and the doxorubicin-resistant line UKF-NB-4$^{DOXO}$ were provided by Dr J. Cinatl Jr (Johann Wolfgang Goethe University, Frankfurt am Mein, Germany). The ellipticine-resistant cell line, designated UKF-NB-4$^{ELLI}$, was established by us by incubating UKF-NB-4 cells with increasing concentrations of ellipticine from 0.2 to 2 μM for one year. Lines were grown in Iscove's modified Dulbeco medium (IMDM; Lonza Inc., Allendale, NJ, USA) supplemented with 10% FCS (PAA Laboratories, Pasching, Austria). The medium for UKF-NB-4$^{DOXO}$ cells contained 100 ng/mL doxorubicin, whereas that for UKF-NB-4$^{ELLI}$ cells contained 2.5 μM ellipticine. The drug resistance of UKF-NB-4$^{ELLI}$ and UKF-NB-4$^{DOXO}$ cells to ellipticine and doxorubicin was verified using the MTT test.[12,16] Resistant cell lines were defined as those showing at least a twofold increase in resistance to ellipticine and doxorubicin. There was cross-resistance between doxorubicin and ellipticine in UKF-NB-4$^{DOXO}$ cells that was not observed between doxorubicin and ellipticine in UKF-NB-4$^{ELLI}$ cells.[6]

**Drugs.** Solutions of doxorubicin (EBEWE Pharma, Unterach, Austria) and ellipticine (Sigma-Aldrich, Deisenhofen, Germany) were prepared according to manufacturers' instructions.

**Cytometry.** To determine protein expression, cells were plated in dishes and treated with ellipticine (0.1, 1.0 and 10.0 μM) for 48 h. Controls were cultured under the same conditions.

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*Membrane lipid packing measurement.* Cells in PBS containing BSA (fatty acid-free; Sigma, St. Louis, MO, USA) were incubated for 10 min with a final concentration of 5 μg/ml of merocyanine 540 (MC540)[17] before being cells washed with phosphate buffered saline/bovine serum albumin (PBS/BSA) and diluted with 500 μl of PBS for flow cytometry (fluorescence intensity measured at 575 nm).

*Antigen detection.* Cells were stained for 15 min with specific antibodies (anti-P-glycoprotein [P-gp] phycoerythrin (PE), anti-CD56 PE, anti-CD57 FITC [Immunotech, Nyon, Switzerland]; anti-lung resistance-related protein (LRP) PE, anti-multiple resistance protein 1 (MRP1) PE, anti-multiple resistance protein 2 (MRP2) PE [IQProducts, Groningen, The Netherlands]), diluted with 500 μl of PBS and measured by FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For the detection of Bcl-2 (anti-Bcl-2 FITC; Caltag, Buckingham, UK), cells were permeabilized with a Fix&Perm kit (Caltag, Buckingham, UK). Expression of above mentioned proteins (P-gp, CD56, CD57, LRP, MRP1, MRP2, Bcl-2) was evaluated as the mean intensity of fluorescence (MIF). The cytometer was calibrated before every measurement. The amount of ellipticine in nuclei was determined using an iCys laser scanning cytometer (CompuCyte, Westwood, MA, USA). Hoechst 34580 was used to segment the nuclei. These segments were then separated according to their size and circularity to represent single nuclei and analyzed later for mean ellipticine fluorescence. At least 1000 segments were used for analysis and samples were measured in triplicate.

**Comparative genomic hybridization.** Genomic DNA was extracted using a Wizard DNA Isolation kit (Promega, Madison, WI, USA) and its concentration was determined using a Biomate3 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). the procedures for CGH used in the present study have been described in detail elsewhere.[13]

**Multicolor FISH.** The mFISH analysis was performed using a 24× Cyte, 24-color whole-chromosome painting probe kit (MetaSystems, Althussheim, Germany) according to the manufacturer's instructions.

**Interphase FISH.** The iFISH analysis was performed per manufacturer's instructions. The probes used for iFISH in the present study are given in Table S1, available as Supporting Information for this paper. Genetic findings from at least 40 cells were used in analyses. To evaluate MYCN (myc myelocytomatosis viral related oncogene, neuroblastoma derived) amplification, 200 cell nuclei were assessed and the number of MYCN copies per cell was determined as the arithmetic mean.

**RNA isolation and real-time RT-PCR.** In the present study, RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of the RNA obtained was controlled using electrophoresis and the quantity was assessed using a Nanodrop 1000 (Thermo Scientific). The RT and PCR conditions used in the present study were as described previously.[6] The reaction mixture contained 2 μL cDNA, 10 μL TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL primer, a 6-carboxy-fluorescein (FAM)-labeled probe mixture of human *CYP1A1*, *CYP1B1*, *CYP3A4*, *COX-1*, *COX-2* and *BCL-2* as target genes and *POLR2A* as the reference gene (Generi Biotech, Hradec Kralove, Czech Republic) and FAM-labeled probe and primers for the human *MYCN* target gene and GAPDH as the reference gene (Applied Biosystems). Samples were analyzed in triplicate. Data were evaluated using the comparative cycle threshold (Ct) method for relative quantitation of expression ($2^{-\Delta\Delta C_T}$).[18]

**Microarray analysis.** Both the parent and resistant cell lines were analyzed in triplicate. A 1-μg aliquot of RNA from each RNA sample was amplified and labeled using an Amino Allyl MessageAmp aRNA kit (Ambion/Applied Biosystems, Austin, TX, USA). The yield of amplification and dye incorporation was checked on a Nanodrop 1000 (Thermo Scientific). We performed two labels balanced experiment; one experiment was done with RNA from sensitive line labeled with cyanine 3, and RNA from resistant line labeled with cyanine 5 and in second experiment, the labeling was performed with opposite labeling (sensitive labeled with cyanine 5, resistant with cyanine 3). Microarray slides (Microarray, Nashville, TN, USA) were prehybridized in buffer with BSA and then 2 μg labeled antisense amplified RNA (aRNA) that underwent fragmentation was hybridized for 16 h at 42°C. The hybridized slides were washed and scanned on a GenePix 4000B (Molecular Devices, Sunnyvale, CA, USA). Image analysis was performed using GenePix Pro 5 software (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed using the R (http://www.r-project.org/, accessed 15 Jan 2009) and limma packages (The Walter and Elisa Hall Institute of Medical Research, Melbourne, Australia).[19–21]

***In vivo* tumorigenicity assay.** Nineteen female CD-1 nude mice were used in these studies. The care and use of animals was in accordance with institutional guidelines. Mice were injected subcutaneously with $1 \times 10^7$ UKF-NB-4 cells ($n = 9$ mice) or UKF-NB-4$^{ELLI}$ cells ($n = 10$ mice) suspended in 100 μL Matrigel (BD, Franklin Lakes, NJ, USA). Every 3 days, the mice were weighed and the tumors were measured and their volume calculated.

**Western blotting.** Protein concentrations were assessed using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). A 10–45-μg sample of protein was subjected to SDS-PAGE. After migration, proteins were transferred to nitrocellulose membranes and incubated with non-fat milk (Bio-Rad). The membranes were exposed to antibodies (see Table S2) overnight at 4°C. Membranes were then washed three times with PBS and once with

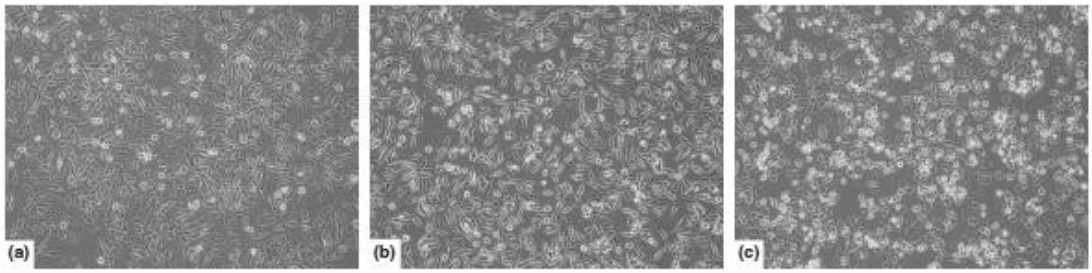


**Fig. 1.** Comparison of the morphology of (a) UKF-NB-4 (parental cell line), (b) ellipticine-resistant UKF-NB-4$^{ELLI}$ and (c) doxorubicin-resistant UKF-NB-4$^{DOXO}$ cells. Phase contrast. Original magnification ×100.

**Table 1.** *In vitro* and *in vivo* growth characteristics of parental and ellipticine-resistant UKF-NB-4 cells

| Cell line | Doubling time *in vitro* | | Tumor volume *in vivo* ($cm^3$) | Tumor formation *in vivo* |
|---|---|---|---|---|
| | Standard conditions | Hypoxic conditions | | |
| UKF-NB-4 | 29.69 ± 0.24 | 32.57 ± 0.75* | 1.365 ± 1.504 | 5/9 |
| UKF-NB-$4^{ELLI}$ | 34.56 ± 0.61† | 45.63 ± 1.60*† | 0.766 ± 0.686 | 3/10 |

Tumor volume and formation were 50 days after subcutaneous injection of tumor cells. There were no significant differences in either parameter between mice injected with UKF-NB-4 and UKF-NB-$4^{ELLI}$ cells. Unless indicated otherwise, data are shown as the mean ± SD of four experiments. *$P < 0.001$ compared with cells grown under standard conditions; †$P < 0.001$ compared with the UKF-NB-4 parental cell line (Student's *t*-test).

Tween/PBS and exposed to HRP-conjugated goat anti-rabbit anti-IgG (H + L) secondary antibodies or HRP-conjugated goat anti-mouse anti-IgG (H + L; Bio-Rad). The antigen–antibody complex was visualized using chemiluminescence (Immun-Star HRP Substrate; Bio-Rad). Films were scanned using an image analyzer (Semecky, Prague, Czech Republic).

**Statistical analysis.** Numerical data are presented as the mean ± SD and were analyzed using Student's *t*-test.

## Results

**Morphology and tumorigenicity of UKF-NB-$4^{ELLI}$ cells.** The UKF-NB-4 cells were incubated for 36 months with increasing concentrations of ellipticine (1–2.5 μM) to induce resistance.[6] After exposure to ellipticine, cells exhibited changes in cell morphology to a fibroblast-like phenotype, with spindle-shaped morphology, the formation of neurite process and a lower cytoplasm : nucleus ratio. This change was observed in most cells (Fig. 1) and was invariable for five passages in ellipticine-free medium. As an ellipticine-resistant cell line, UKF-NB-$4^{DOXO}$ cells did not exhibit any marked changes, with preservation of the characteristics of S-type NBL but with more oval-shaped cells and a less flat cytoplasm.

The growth rate of UKF-NB-$4^{ELLI}$ cells was slower than that of UKF-NB-4 cells, with doubling times of 34.4 ± 0.6 *vs* 26.7 ± 0.2 h, respectively. The levels of drug resistance of lines UKF-NB-$4^{ELLI}$ and UKF-NB-$4^{DOXO}$ compared with UKF-NB-4 expressed as $IC_{50}$ were described.[6] There were differences in the growth of UKF-NB-4 and UKF-NB-$4^{ELLI}$ cells *in vivo* (Table 1). Specifically, UKF-NB-$4^{ELLI}$ cells exhibited lower tumorigenicity and slower growth *in vivo* compared with UKF-NB-4 cells (Fig. 2a). In the case of UKF-NB-4 cells, tumors grew in five of nine animals injected with these cell, whereas only three of 10 mice injected with UKF-NB-$4^{ELLI}$ cells had tumors. The weights of mice with UKF-NB-$4^{ELLI}$ cells were higher than those of mice with UKF-NB-4 (Fig. 2b).

**Genomic imbalances in UKF-NB-$4^{ELLI}$ cells.** Genetic changes in ''preresistant UKF-NB-$4^{ELLI}$'' cells (UKF-NB-4 cells resistant to 1.5 μM ellipticine, 50 passages with ellipticine) and resistant UKF-NB-$4^{ELLI}$ cells (resistant to 2.5 μM ellipticine, 100 passages) were examined using CGH to exclude changes that may have been acquired during culture.[22] No changes were detected in individual chromosome levels in these two lines. After CGH analyses, mFISH was used to detect balanced aberrations and iFISH was used to verify genetic changes (Tables S3, S4; Fig. 3). Except for 2p24 (*MYCN*), there was a decrease in the copy number of a chromosomal gain on chromosome 7 in UKF-NB-$4^{ELLI}$ cells. The UKF-NB-4 gained a chromosome 7, whereas UKF-NB-$4^{ELLI}$ cells lost 7q11.2–31.3 (Fig. 4a). For verification of changes at chromosome 17, iFISH probes p53 (17p13)/MPO (17q23) and TOP2A (17q21) were used to describe the areas in which genes related to drug resistance are located. The UKF-NB-4 cells had a TP53 deletion, whereas UKF-NB-$4^{ELLI}$ cells had three copies of TP53. The UKF-NB-4 cells had five copies of the *MPO* gene, whereas the UKF-NB-$4^{ELLI}$ cells lost one copy, but still had a 17q23 gain (Fig. 4b). Furthermore, mFISH analyses revealed disomy of chromosome 17 and other parts of chromosome 17 translocated onto chromosomes 1 and 10 (Fig. 3).

**Expression characteristics of UKF-NB-$4^{ELLI}$ cells.** The chromosome expression profile was detected using microarrays (Fig. S1). The datasets were further subjected to significance analysis of microarrays (differences of gene expression between UKF-NB-$4^{ELLI}$ and UKF-NB-4) to highlight candidate genes induced by prolonged ellipticine treatment. One of the mechanisms that may explain resistance to ellipticine in UKF-NB-$4^{ELLI}$ cells is downregulation of TOP2A and TOP1 (Fig. 5). Further analysis using qRT-PCR confirmed downregulations that corresponded to the loss of one gene copy of TOP1 and TOP2A, demonstrated using iFISH (Fig. 4b). Differences in mRNA expression of enzymes involved in the metabolism of ellipticine (*CYP1A1*, *CYP3A4*, *COX-1* and *COX-2*; Table S5) were not found by microarray. Conversely, using qRT-PCR, a twofold increase in the mRNA expression of *CYP3A4* and *COX-1* was found in UKF-NB-$4^{ELLI}$ cells (Table 2). Western blotting revealed decreased expression of CYP1B1 protein in UKF-NB-$4^{ELLI}$ cells, whereas the expression of other enzymes correspond

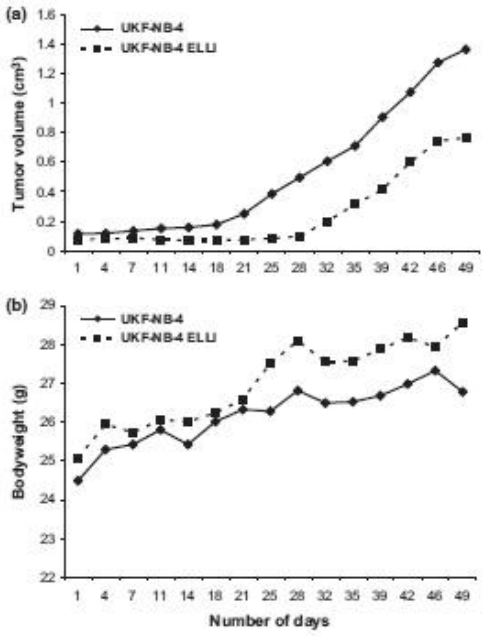


**Fig. 2.** (a) *In vivo* growth of tumors and (b) the body weight of mice with injected neuroblastoma cells.



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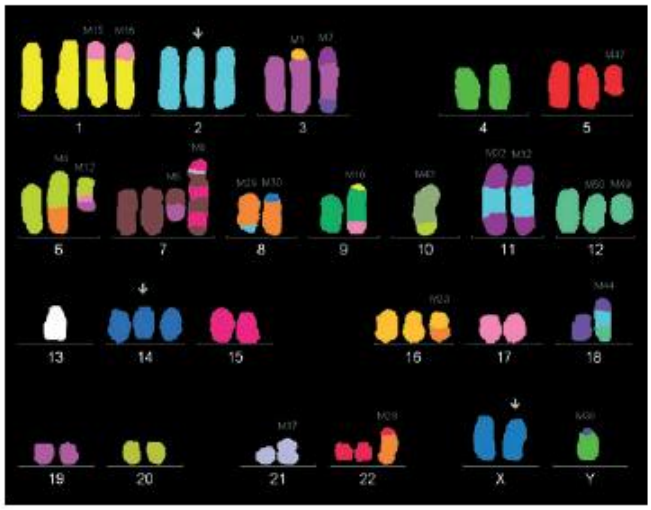

Fig. 3. Karyotype of the UKF-NB-4$^{ELLI}$ cell line evaluated by multicolor FISH. A list of marker chromosomes is given in Table S4.

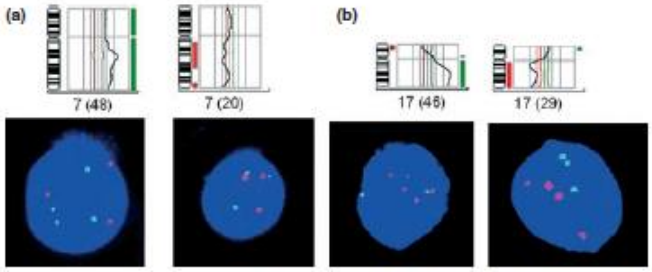


Fig. 4. (a) Comparative genomic hybridization (CGH) profile of chromosome 7 in UKF-NB-4 and UKF-NB-4$^{ELLI}$ cells. The CGH analysis of UKF-NB-4 cells revealed a whole chromosome gain of chromosome 7 with higher-level gain of 7q21.1–31.1 (four green signals on the FISH picture), whereas in UKF-NB-4$^{ELLI}$ cells there was a loss of gain of 7q11.2–q21.3 (three green signals) compared with the parental chromosome 7. (b) The CGH profile of chromosome 17 in UKF-NB-4 and UKF-NB-4$^{ELLI}$ cells. The CGH analysis of UKF-NB-4 cells revealed a loss of 17p13 (two red signals) and a gain of 17q (five green signals), whereas in UKF-NB-4$^{ELLI}$ cells one lost copy of 17p13 is restored (three red signals) and the gain of 17q is partially lost (four red signals).

to the results obtained for mRNA expression (Fig. 6). In addition, there was a significantly upregulation of the gene expression of proteins associated with DNA repair, especially nucleotide-excision repair (Table S5) and oxidative phosphorylation, cell respiratory chain and aerobic respiration enzymes (Table S6). In contrast, significant downregulation was found for genes encoding double-strand break repair proteins (Table S5).

Microarray analysis revealed a tendency for decreased *MYCN* expression in UKF-NB-4$^{ELLI}$ cells, although the difference failed to reach statistical significance (Table S5). This decrease in *MYCN* mRNA was confirmed using qRT-PCR and was found to be significant ($P < 0.001$, *t*-test). Although the expression of *MYCN* mRNA was higher in UKF-NB-4$^{ELLI}$ cells cultured in the presence of ellipticine than in its absence, its expression remained lower than in UKF-NB-4 cells. Nevertheless, *MYCN* gene expression in UKF-NB-4$^{ELLI}$ cells remained higher than in the NBL line without *MYCN* amplification (SK-N-AS) or in leukocytes (data not shown). Levels of the N-myc protein corresponded to mRNA levels (Table 2; Fig. 6).

Expression of the regulatory members of apoptotic pathways, namely BAX and BCL-2, analyzed using microarray, did not change significantly, but qRT-PCR revealed that the expression BCL-2 was twofold greater in UKF-NB-4$^{ELLI}$ cells than in UKF-NB-4 cells (Table 2). Western blotting showed no change in Bax and an increase in Bcl-2 expression in UKF-NB-4$^{ELLI}$ cells (Fig. 6).

**Multidrug-resistance phenotype of UKF-NB-4$^{ELLI}$ cells compared with UKF-NB-4$^{DOXO}$ cells.** During cytometric analysis, we observed an increase in the intensity of ellipticine fluorescence in resistant cells cultivated for 1 h in lower concentrations of 0.1 and 1 μM of ellipticine, but not at the highest concentration of 10 μM (Fig. 7a). There was a direct relationship between UKF-NB4 cells cultured in the presence of higher concentrations of 1 and 10 μM of ellipticine and higher nuclear fluorescence (Fig. 7b; 18 h culture). Cytometric analysis of P-gp detected a significant decrease in P-gp expression in UKF-NB-4$^{ELLI}$ cells compared with UKF-NB-4 cells, whereas in UKF-NB-4$^{DOXO}$ P-gp expression was more than threefold greater than in UKF-NB-4 cells (Table 3). Increased expression of LRP, MRP, and Bcl-2

proteins, measured using cytometry, was detected in UKF-NB-4$^{EL.LI}$ cells. Those antigens, as well as MRP1, were also overexpressed in UKF-NB-4$^{DOXO}$ cells. Decreased expression of NCAM (CD56) was detected in both drug-resistant lines (i.e. UKF-NB-4$^{EL.LI}$ and UKF-NB-4$^{DOXO}$) compared with the parent cell line. The level of lipid packing, evaluated as the MIF of MC540-stained cells, was 111.9 ± 2.3, 245.8 ± 1.1, and 89.4 ± 3.2 in UKF-NB-4, UKF-NB-4$^{EL.LI}$, and UKF-NB-4$^{DOXO}$ cells, respectively. This illustrates that, in UKF-NB-4$^{DOXO}$ cells, there was increased packing of the lipid bilayer compared with UKF-NB-4 cells, whereas in UKF-NB-4$^{EL.LI}$ cells it was the opposite.

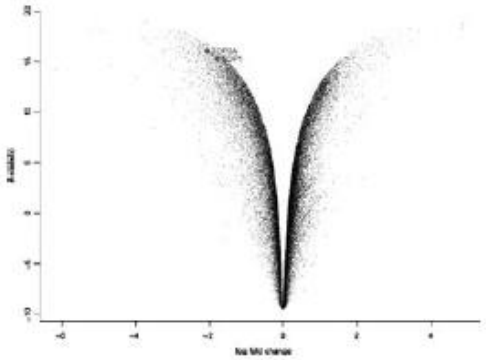

**Fig. 5.** Volcano plot of gene expression between cells resistant to ellipticine and the parental cells. The *x*-axis shows fold changes on a logarithmic scale (base 2), whereas the *y*-axis shows the Bayesian (B) statistic.[19,20] The expression of topoisomerases TOP2A and TOP1 is highlighted.

## Discussion

The mechanisms underlying drug resistance are: (i) increased drug efflux; (ii) downregulation, overexpression, or modification of target molecules; (iii) changes in drug activating and/or detoxifying enzymes; (iv) the induction of anti-apoptotic mechanisms or the inactivation of pro-apoptotic mechanisms; and (v) pharmacological and physiological factors, such as changes in drug metabolism and excretion, and inadequate access of the drug to the tumor.[23] Cell lines have genetic abnormalities because they have been derived from tumors. Lines with induced drug resistance are used to investigate the changes accompanying drug resistance regardless of genetic abnormalities caused by multiple passages and/or by cytostatics, which are not related to drug resistance.[13,24,25]

Cytogenetics of drug-resistant lines cannot determine which abnormalities occurred in the selection process and which are intrinsic to the parental line. Therefore, we hybridized DNA from the resistant line and compared it with that of the parental line in CGH, thus identifying only genetic abnormalities specific to the selection process only.[26] For detections of balanced aberrations we used mFISH. Genetic aberrations were verified using iFISH. On chromosome 2, we found a loss of 2p21-pter. The UKF-NB-4$^{EL.LI}$ cells lost 24 *MYCN* copies compared with UKF-NB-4 cells. This phenomenon of lost *MYCN* copies in drug-resistant cells we explain as expulsion of amplified *MYCN* caused by cytostatic drugs.[27] Both preresistant UKF-NB-4$^{EL.LI}$ and resistant UKF-NB-4$^{EL.LI}$ cells had the same *MYCN* copy number. This finding supports the argument that loss of *MYCN* copies occurs during the first run of culture with the cytostatic drug and then remains stable.[27] We can conclude that nearly all

**Table 2. Expression of mRNA related to ellipticine activation and apoptotic pathways in the parental and ellipticine-resistant sublines**

| Cell line | $2^{-\Delta\Delta C_T}$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | *MYCN* | *CYP1A1* | *CYP1B1* | *CYP3A4* | *COX-1* | *COX-2* | *BCL-2* |
| UKF-NB-4 | 1.00 ± 0.13 | 1.00 ± 0.21 | 1.00 ± 0.18 | 1.00 ± 0.22 | 1.00 ± 0.27 | 1.00 ± 0.16 | 1.00 ± 0.12 |
| UKF-NB-4$^{EL.LI}$ | 0.46 ± 0.06 | 0.88 ± 0.18 | 0.32 ± 0.06 | 1.80 ± 0.78 | 2.01 ± 0.79 | 0.55 ± 0.17 | 2.17 ± 0.28 |

Data are the mean ± SD of three experiments and are expressed as fold changes compared with the sensitive cell line.

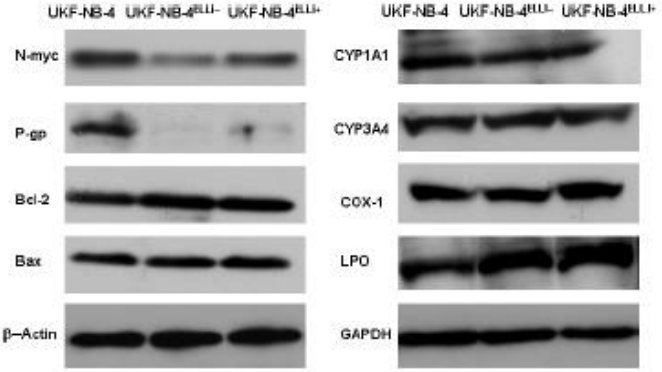


**Fig. 6.** Immunoblots of N-myc, P-glycoprotein (P-gp), Bcl-2, Bax, cytochrome P450 (CYP) 1A1, CYP3A4, Cox-1 and lactoperoxidase (LPO) in UKF-NB-4 and UKF-NB-4$^{EL.LI}$ cells. β-Actin and GAPDH were used as internal controls. UKF-NB-4, sensitive cell line cultured in the absence of ellipticine; UKF-NB-4$^{EL.LI-}$, resistant cell line cultured in the absence of ellipticine; UKF-NB-4$^{EL.LI+}$, resistant line cultured in the presence of ellipticine.



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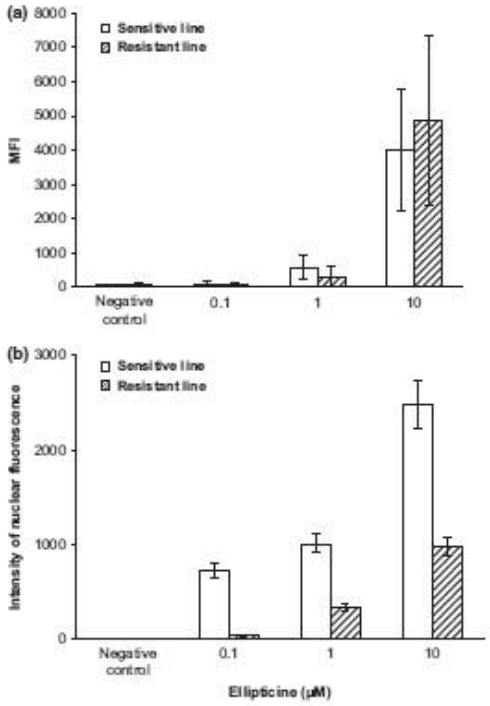


**Fig. 7.** (a) Mean fluorescence intensity (MFI) of cell lines incubated for 1 h with ellipticine, as determined by flow cytometry. (b) Nuclear fluorescence intensity measured by laser scanning cytometry. Cells were incubated for 18 h in the presence of ellipticine.

**Table 3. Expression of proteins related to cell resistance in parental, doxorubicin- and ellipticine-resistant UKF-NB-4 cells**

| Protein | Relative MIF | | |
|---|---|---|---|
| | UKF-NB-4 | UKF-NB-4$^{ELLI}$ | UKF-NB-4$^{DOXO}$ |
| P-gp | 1.000 ± 0.004 | 0.486 ± 0.030 | 3.418 ± 0.167 |
| LRP | 1.000 ± 0.037 | 1.472 ± 0.010 | 1.410 ± 0.124 |
| MRP1 | 1.000 ± 0.032 | 0.975 ± 0.009 | 1.170 ± 0.055 |
| MRP2 | 1.000 ± 0.040 | 1.403 ± 0.147 | 1.408 ± 0.044 |
| CD56/NCAM | 1.000 ± 0.012 | 0.871 ± 0.070 | 0.731 ± 0.046 |
| CD57 | 1.000 ± 0.011 | 1.713 ± 0.144 | 2.459 ± 0.144 |
| Bcl-2 | 1.000 ± 0.014 | 1.060 ± 0.015 | 1.386 ± 0.098 |

Values are mean ± SD of at least three experiments. Results are shown as the relative mean intensity of fluorescence (MIF), where the MIF of the UKF-NB-4 cell line was set as 1 and the relative MIF of the doxorubicin- and ellipticine-resistant UKF-NB-4 cell lines (UKF-NB-4$^{DOXO}$ and UKF-NB-4$^{ELLI}$, respectively) were calculated using the formula Relative MIF = $MIF_{resistant\ line}/MIF_{UKF\text{-}NB\text{-}4}$. P-gp, P-glycoprotein; LRP, lung resistance-related protein; MRP1, multiple resistance protein 1; MRP2, multiple resistance protein 2; NCAM, neural cell adhesion molecule (CD56).

chromosome changes were identified using CGH and mFISH. Differences between mFISH and iFISH or CGH may be explained by minority cell populations that can be detected in mitosis using mFISH. Note that mFISH describes not only changes in the number of chromosomes, but also translocations and inversions. A combination of these techniques seems to be suitable for the detection of genomic aberrations in cell lines. In the present study, iFISH was used as a method to confirm the exact number of gene copies or numbers of chromosome arms (i.e. as verification of CGH and/or mFISH results).

The gain of 7q11.2–q21.3 in UKF-NB-4 cells and the loss of 7q11.2–q21.3 in UKF-NB-4$^{ELLI}$ cells prompted us to speculate that the *MDR1* gene does not cause ellipticine resistance. The *MDR1* gene encodes P-gp, a member of the ABC transporter superfamily. Expression of *MDR1* mRNA and P-gp in UKF-NB-4$^{ELLI}$ cells was lower than in UKF-NB-4 cells (Fig. 6). Moreover, array analysis indicated that expression of most ABC transporters was unchanged in UKF-NB-4$^{ELLI}$ cells. Studies of the mechanism of doxorubicin resistance in our laboratory indicate that amplification of 7q21 (*MDR1* gene)[13] has importance in doxorubicin resistance. The UKF-NB-4$^{ELLI}$ cells show fewer genetic changes than UKF-NB-4$^{DOXO}$ cells. Conversely, some changes were the same for both resistant lines. Increased expression of LRP and MRP2 proteins, found in UKF-NB-4$^{ELLI}$ and UKF-NB-4$^{DOXO}$ cells, may participate in the expulsion of drugs. Both MRP1 and, in particular, P-gp are important in the resistance to doxorubicin;[28] neither was overexpressed in UKF-NB-4$^{ELLI}$ cells and overexpression was noted only in UKF-NB-4$^{DOXO}$ cells. Accumulation of ellipticine in the nuclei of UKF-NB-4$^{ELLI}$ cells was decreased, as determined using laser scanning cytometry, whereas total fluorescence of UKF-NB-4, as determined by flow cytometry after exposure of the cells to 10 μM ellipticine, was not significantly lower than in UKF-NB-4$^{ELLI}$ cells. We suppose that intracellular sequestration and/or degradation may play role in ellipticine resistance rather than increased efflux. The hypothesis that intracellular sequestration of ellipticine plays a role in drug resistance is supported by increased expression of V-ATPases. It has been reported that ATPase subunits are upregulated in drug resistant cell lines.[29,30] The extracellular pH in solid tumors is significantly more acidic than in normal tissue. This increased acidity interferes with the absorption of basic chemotherapeutic drugs, reducing their effect on tumors. It is believed that V-ATPase is largely responsible for the regulation of the acidity of the tumor microenvironment.[31] The induction of V-ATPase expression also has anti-apoptotic effects and V-ATPase inhibitors (e.g. omeprazole) in combination with cytostatics may offer a new therapeutic approach.

Increased Bcl-2 protein expression that suppresses caspase activation may also be important; it has been associated with drug resistance in several tumors. Preclinical studies have shown that agents targeting Bcl-2 have anticancer activity, particularly in combination with other antineoplastic agents.[32] Interestingly, the expression of CYP3A4 and COX-1, enzymes involved in the metabolism of ellipticine, was higher in UKF-NB-4$^{ELLI}$ cells. Surprisingly we detected low expression of CYP1B1 protein, which, with CYP1A1 and 1A2, detoxifies ellipticine.[33]

The decreased expression of CD56 found in UKF-NB-4$^{ELLI}$ cells has also been described in doxorubicin- and vincristine-resistant NBL cell lines.[34] Decreased expression of CD56 results in increased adhesion and transendothelial penetration, which increases the ability to metastasize. Drugs do not move across the cell membrane freely and the status of the plasma membrane may contribute to drug resistance.[35] The accumulation of drugs in cells with P-gp could be accompanied by changes in membrane properties.[36] Increased binding of MC540 is a consequence of the probe's property of binding more strongly to a loosely packed lipid bilayer than to a more tightly packed layer.[17] Reduced packing of the membrane lipids of sensitive A549 cells compared with a cisplatin-resistant subline has been reported.[37] We found similar results in the parental NBL lines UKF-NB-3 and UKF-NB-4 and the doxorubicin- and cisplatin-resistant sublines derived from them (Eckschlager T, unpublished observation, 2010). Therefore, the

loosely packed lipid bilayer in UKF-NB-4$^{ELLI}$ cells was a surprise, but it may be connected to the decreased expression of P-gp.

We found that the spermine synthetase gene was upregulated (Table S5). This gene encodes the enzyme that catalyses the conversion of putrescine to spermidine. Spermine, formed from spermidine, stabilizes the helical structure of nucleic acids. Our finding supports the idea that upregulation of DNA repair mechanisms could play a role in ellipticine resistance. It has been reported that spermine deficiency sensitizes cells to the antiproliferative effects of etoposide[38] and bis-chloroethylnitrosourea (BCNU).[39] This may explain why increased spermine synthetase may decrease the efficiency of ellipticine, which acts as a topoisomerase inhibitor similar to etoposide. Polyamines have been described to be associated with drug resistance.[40] Doxorubicin and ellipticine act by inhibiting TOP2. There are two mechanisms of resistance to TOP2 inhibitors: (i) changes TOP2 activity; or (ii) gene mutation.[41] In the present study, we found decreased expression of TOP1 and TOP2A.

Levels of DNA adducts were correlated with the toxicity of ellipticine against NBL cells. The main adducts were Adducts 1 and 2 derived from 13-hydroxy- and 12-hydroxyellipticine, respectively. Two additional adducts, namely Adducts 6 and 7, formed in DNA by ellipticine activated by peroxidases, were also detected after incubation with 10 μM ellipticine, but not with 1 μM ellipticine.[7] In UKF-NB-4$^{ELLI}$ cells, adduct levels were lower than levels in the parental cell line after incubation with the same concentration of ellipticine.[6] The reason for this phenomenon remains unknown. The slower growth of UKF-NB-4$^{ELLI}$ cells may also be a factor in resistance.

Partial cross-resistance between ellipticine and doxorubicin may be important in clinical practice.[6] One can speculate that physicians will have regard for this cross-resistance in preparing therapeutic protocols involving doxorubicin and ellipticine.

In conclusion, resistance to ellipticine in NBL is caused by a combination of overexpression of Bcl-2, efflux or degradation of the drug, and downregulation of topoisomerases. Upregulation of enzymes of oxidative phosphorylation, cellular respiration, V-ATPases, aerobic respiration and/or spermine synthetase and slower growth may also contribute to resistance. Some changes are expressed at the DNA level. The present study established that cancer cell drug resistance is caused by a combination of mechanisms and, to overcome this problem, will require a complex approach.

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## Disclosure Statement

The authors have no conflicts of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Changes in mRNA expression in UKF-NB-4$^{EL1.1}$ compared with UKF-NB-4 cells as detected by microarray.

**Table S1.** List of probes used in the iFISH.

**Table S2.** List of antibodies used for western blot analysis.

**Table S3.** Karyotype abnormalities in the parental and ellipticine-resistant sublines.

**Table S4.** List of marker chromosomes detected by mFISH.

**Table S5.** Analysis of array datasets for genes implicated in the acquired ellipticine resistance phenotype.

**Table S6.** Analysis of array datasets indicated predominantly statistically high significant (≥10 in cell line UKF-NB-4$^{EL1.1}$ than in cell line UKF-NB-4) upregulated genes in the oxidative phosphorylation.

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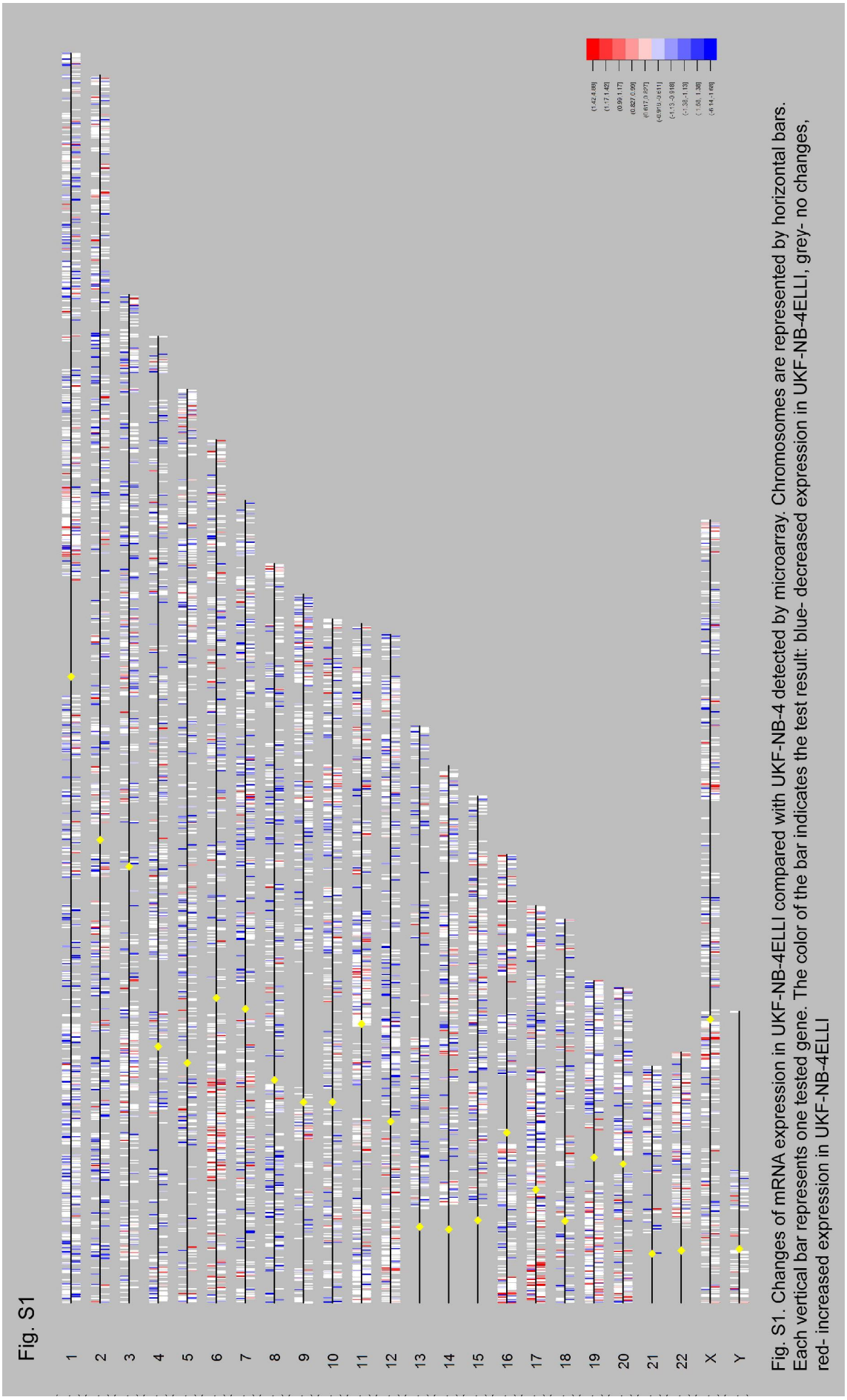


Fig. S1. Changes of mRNA expression in UKF-NB-4ELLI compared with UKF-NB-4 detected by microarray. Chromosomes are represented by horizontal bars. Each vertical bar represents one tested gene. The color of the bar indicates the test result: blue- decreased expression in UKF-NB-4ELLI, grey- no changes, red- increased expression in UKF-NB-4ELLI

**Table S1. List of used iFISH probes**

| chromosome | iFISH probe | manufacturer |
|---|---|---|
| 1 | ON 1p36 / SE 1 | Kreatech |
| 1 | ON 1q21 / SRD (1p36) | Kreatech |
| 1 | ON MDM4 (1q32) / SE 1 | Kreatech |
| 2 | ON MYCN (2p24) / LAF | Kreatech |
| 3 | ST 3pter | Kreatech |
| 3 | SE 3 (D3Z1) | Kreatech |
| 6 | SE 6 (D6Z1) | Kreatech |
| 7 | ON EGFR, Her-1 (7p11) / SE 7 | Kreatech |
| 7 | ON MDS 7q- (7q22; 7q35) | Kreatech |
| 8 | LSI C-MYC | Vysis |
| 8 | Chromosome 8 alpha satellite | Cytocell |
| 9 | ST 9pter | Kreatech |
| 9 | ON p16 (9p21) / SE 9 | Kreatech |
| 10 | ST 10pter | Kreatech |
| 10 | SE 10 (D10Z1) | Kreatech |
| 11 | ON ATM (11q23) / SE 11 | Kreatech |
| 12 | ON GLI (12q13) / SE 12 | Kreatech |
| 12 | ON MDM2 (12q15) / SE 12 | Kreatech |
| 13, 21 | SE 13 / 21 | Kreatech |
| 13 | LSI 13 / RB1 | Vysis |
| 14, 18 | ON BCL2/IGH t(14;18) Fusion | Kreatech |
| 16 | ST 16pter | Kreatech |
| 16 | ST 16qter | Kreatech |
| 17 | ON p53 (17p13) / MPO (17q23) "ISO 17q" | Kreatech |
| 17 | ON ERBB2, Her-2/neu (17q12) / SE 17 | Kreatech |
| 17 | ON TOP2A (17q21) / SE 17 | Kreatech |
| 19 | ST 19pter | Kreatech |
| 19 | ST 19qter | Kreatech |
| 20 | ST 20pter | Kreatech |
| 20 | ST 20qter | Kreatech |
| 22 | ST 22qter | Kreatech |
| X | SE X (DXZ1) | Kreatech |
| Y | SE Y (DYZ3) | Kreatech |

Kreatech, Kreatech Biotechnology B. V., Amsterdam, Netherlands; Vysis, Vysis, Downers Grove, IL, USA; Cytocell, Cytocell Ltd., Cambridge, UK.

**Table S2. List of antibodies used for western blot**

| Antigene | Dilution | Producer |
|---|---|---|
| CYP1A1 | 1 : 1 000 | Millipore, Billerica, USA |
| CYP1B1 | 1 : 1 000 | AbCam, USA |
| CYP3A4 | 1 : 2 500 | AbD Serotec, Oxford, UK |
| LPO | 1 : 2 000 | AbCam, MA, USA |
| Bax | 1 : 750 | Cell Signaling, Danvers, USA |
| Cyb5 | 1 : 750 | AbCam, USA |
| P-gp | 1 : 800 | Millipore, Billerica, USA |
| Cox-1 | 1 : 2 000 | AbCam, Cambridge, UK |
| Cox-2 | 1 : 1 000 | Exalpha Biologicals, Shirley, USA |
| N-myc | 1 : 20 000 | BD Pharmingen, Franklin Lakes, USA |
| bcl-2 | 1 : 1 000 | Millipore, Billerica, USA |
| actin | 1 : 3 000 | Sigma-Aldrich, Deisenhofen, Germany |
| GAPDH | 1 : 750 | Millipore, Billerica, USA |

**Table S3.** Karyotype abnormalities in the parental and the ellipticine-resistant subline

| Parental cell line<br>UKF-NB-4 | chromosome number | karyotype method | Ellipticine-resistant cell line<br>UKF-NB-4ELLI |
|---|---|---|---|
| -1p32.2-pter, +1p22.3-qter, **+1q25-42.1** | 1 | CGH | -1p35-pter* |
| NA | | mFISH | +der(1)t(1;17)(p21;?),+der(1)t(1;17)(p21;?) |
| 2x1p36.31, 4x1qh, 4x1q21, 8x32.1 | | iFISH | 2x1p36, 4x1qh, 4x1q21, 7x32.1, 8x32.1 |
| +2, **+2p13-pter** | 2 | CGH | -2p21-pter* |
| NA | | mFISH | +2 |
| 66x2p24.3, 4x2q11.2 | | iFISH | 42x2p24.3, 4x2q11.2 |
| -3p21.1-pter, +3p14.1-qter, **+3q26.1-qter** | 3 | CGH | -3q24-qter* |
| NA | | mFISH | der(3)t(3;16)(p24.1;?),+der(3)t(3;11)(p14.3;q?)t(3;18)(q26.1;q12) |
| 2x3pter, 3xD3Z1 | | iFISH | 2x3pter, 3xD3Z1 |
| 0 | 4 | CGH | -4p16* |
| NA | | mFISH | - |
| NA | | iFISH | NA |
| **+5** | 5 | CGH | 0* |
| NA | | mFISH | i(5)(p10),+del(5)(q?) |
| NA | | iFISH | NA |
| 0 | 6 | CGH | +6q14-pter* |
| NA | | mFISH | der(6)t(6;8)(q22.1;q?),+der(6)t(3;6;17)(?;q12;?) |
| 2xD6Z1 | | iFISH | 3xD6Z1 |
| +7, **+7q21.1-31.1** | 7 | CGH | -7q11.2-31.3, -7q36* |
| NA | | mFISH | der(7)t(7;19)(p11.2;?),+der(7)t(7;15;21)(p13;?;?)ins(7;15)(q11.2;q?)ins(7;15)(q?;q?) |
| 4x7p11.2, 4xD7Z1, 4x7q22, 3x7q35 | | iFISH | 4x7p11.2, 4xD7Z1, 3x7q22, 3x7q35 |
| +8, **+8p** | 8 | CGH | -8q11.2-pter*, -8q22-qter* |
| NA | | mFISH | der(8)t(8;18)(q11.2;?),der(8)t(2;8)(?;q22),der(8)t(X;8)(?;p12) |
| 4xD8Z2, 3x8q24.21 | | iFISH | 3xD8Z2, 2x8q24.21 |

| | | | |
|---|---|---|---|
| -9q34.1-pter | | CGH | -9q34.1-qter* |
| NA | 9 | mFISH | der(9)t(1;9)(?;p36.3)t(9;17)(q34;?) |
| 1x9pter, 2x9p21, 2x9q12 | | iFISH | 1x9pter, 2x9p21, 2x9q12 |
| -10 | | CGH | -10p13-pter* |
| NA | 10 | mFISH | der(10)t(6;10)(?;q25),-10 |
| 3x10pter, 2xD10Z1 | | iFISH | 2x10pter, 2xD10Z1 |
| -11p, +11q13.4-qter | | CGH | -11q13.2-14.1* |
| NA | 11 | mFISH | der(11)ins(11;2)(p11.1;?) x2 |
| 2xD11Z1, 3x11q23 | | iFISH | 2xD11Z1, 3x11q23 |
| +12 | | CGH | +12p*,-12q12-21.1* |
| NA | 12 | mFISH | del(12)(p12) x2, +der(12)del(12)(p12)del(12)(q22) |
| 3xD12Z3, 3x12q13 | | iFISH | 3xD12Z3, 2x12q13, 2x12q15 |
| -13q14.11-qter | | CGH | 0* |
| NA | 13 | mFISH | - |
| 5x13+21, 2x13q14.2 | | iFISH | 4x13+21, 2x13q14.2 |
| +14 | | CGH | 0* |
| NA | 14 | mFISH | +14 |
| 3x14q32.33 | | iFISH | 3x14q32.33 |
| **+15** | | CGH | 0* |
| NA | 15 | mFISH | - |
| NA | | iFISH | NA |
| +16p,-16q | | CGH | -16p* |
| NA | 16 | mFISH | der(16)t(8;16)(p12;p11.1) |
| 4x16pter, 2x16qter | | iFISH | 3x16pter, 2x16qter |
| -17p12-pter, +17q, **+17q11.1-qter** | | CGH | +17p12-pter, -17q |
| NA | 17 | mFISH | - |
| 2x17p13, 3xD17Z1, 4x17q12, 5x17q21, 5x17q22 | | iFISH | 3x17p13, 3xD17Z1, 3x17q12, 3x17q21, 4x17q22 |
| +18q, **+18q12.3-qter** | | CGH | -18q21.1-qter* |
| NA | 18 | mFISH | - |
| 4x18q21.33 | | iFISH | 3x18q21.33 |

| | | | |
|---|---|---|---|
| 0 | | CGH | -19q* |
| NA | 19 | mFISH | - |
| 3x19pter, 3q19qter | | iFISH | 3x19pter, 2xq19qter |
| +20 | | CGH | -20* |
| NA | 20 | mFISH | - |
| 3x20pter, 3x20qter | | iFISH | 2x20pter, 2x20qter |
| +21 | | CGH | -21* |
| NA | 21 | mFISH | i(21)(q10) |
| 5x13+21 | | iFISH | 4x13+21 |
| -22 | | CGH | -22q13.1-qter* |
| NA | 22 | mFISH | +der(22)t(8;22)(q11.2;q11.1) |
| 3x22qter | | iFISH | 3x22qter |
| +X | | CGH | 0* |
| NA | X | mFISH | +X |
| 2xDXZ1 | | iFISH | 2xDXZ1 |
| 0 | | CGH | 0* |
| NA | Y | mFISH | der(Y)t(Y;4)(q11.22;q?) |
| 1xDYZ3 | | iFISH | 1xDYZ3 |

CGH analysis indicated all copy gains (+) and losses (-) in the parental cell line and only new genomic imbalances in ellipticine-resistant subline in comparison to parental cell line. Bold type indicates amplifications. 0, represents chromosome with no CGH change. *, describes only new chromosome changes in UKF-NB-4$^{ELLI}$, other changes are still presented as in parental line. 0*, it indicates the same result as in UKF-NB-4. mFISH analysis indicated karyotype imbalances in the UKF-NB-4$^{ELLI}$ subline. - no indicated karyotype changes in mentioned chromosome. iFISH analysis indicated number of chromosome bands according to number of locus specific areas that emitted light using specific fluorescence labeled DNA probe. NA, not analyzed.

**Table S4. List of marker chromosomes detected by mFISH**

| | | |
|---|---|---|
| **M 1** | **der(3)t(3;16)(p24.1;?)** | **9x** |
| **M 2** | **der(3)t(3;11)(p14.3;q?)t(3;18)(q26.1;q12)** | **6x** |
| **M 3** | der(18)t(12;18)(q11.2;q?) | 1x |
| **M 4** | **der(6)t(6;8)(q22.1;q?)** | **4x** |
| **M 5** | der(6)t(6;8)(q22.1;q?)t(X;6)(p21;?) | 1x |
| **M 6** | **der(7)t(7;19)(p11.2;?)** | **5x** |
| **M 7** | der(7)t(3;7)(?;q11.2) | 1x |
| **M 8** | **der(7)t(7;15;21)(p13;?;?)ins(7;15)(q11.2;q?)ins(7;15)(q?;q?)** | **11x** |
| **M 9** | **der(8)t(8;18)(q11.2;?)** | **4x** |
| **M 10** | **der(9)t(1;9)(?;p36.3)t(9;17)(q34;?)** | **10x** |
| **M 11** | der(14)t(4;14)(?;q32) | 2x |
| **M 12** | **der(6)t(3;6;17)(?;q12;?)** | **8x** |
| **M 13** | der(17)t(13;17)(q?;q22) | 2x |
| **M 14** | der(16)t(16;22)(p12;q?)del(16)(q12.1) | 1x |
| **M 15** | **der(1)t(1;17)(p21;?)** | **10x** |
| **M 16** | der(5)add(5)(p?)t(5;11)(q32;?) | 2x |
| **M 17** | der(6)t(X;6)(?;q27) | 2x |
| **M 18** | der(2)t(2;11)(p13;q?)t(2;11)(q31;?) | 1x |
| **M 19** | ider(19)t(19;21)(q13.3;q?) | 1x |
| **M 20** | der(20)t(8;20)(?;q13.3) | 3x |
| **M 21** | der(22)t(1;5;22)(?;?;p11.1) | 1x |
| **M 22** | der(X)t(X;5)(p22.1;?) | 2x |
| **M 23** | dic(X)t(X;13)(p22.1;p11.1) | 1x |
| **M 24** | der(6)t(6;8)(q22.1;?)t(6;17)(p21.3;?) | 1x |
| **M 25** | der(17)t(17;18)(p11.1;q11.2) | 1x |

| | | |
|---|---|---|
| **M 26** | der(3)t(3;18)(q26.1;q12)ins(3;11)(p13;?) | 2x |
| **M 27** | der(22)t(12;22)(?;p11.1) | 1x |
| **M 28** | **der(22)t(8;22)(q11.2;q11.1)** | **4x** |
| **M 29** | **der(8)t(2;8)(?;q22)** | **3x** |
| **M 30** | **der(8)t(X;8)(?;p12)** | **4x** |
| **M 31** | der(9)t(1;9;11)(?;q21;?) | 1x |
| **M 32** | **der(11)ins(11;2)(p11.1;?)** | **8x** |
| **M 33** | **der(16)t(8;16)(p12;p11.1)** | **4x** |
| **M 34** | der(10)t(5;10)(p12;q11.1) | 2x |
| **M 35** | der(1)t(1;15)(p21;q25) | 1x |
| **M 36** | **der(Y)t(Y;4)(q11.22;q?)** | **9x** |
| **M 37** | **i(21)(q10)** | **7x** |
| **M 38** | der(21)t(19;21)(q?;q21) | 1x |
| **M 39** | der(1)t(1;2;17)(p21;?;?) | 1x |
| **M 40** | der(12)t(2;12;15)(?;q24.1;?) | 1x |
| **M 41** | dic(2;13)(?;q33) | 1x |
| **M 42** | der(2)t(2;17)(q22;?) | 2x |
| **M 43** | **der(10)t(6;10)(?;q25)** | **3x** |
| **M 44** | der(18)t(2;12;18)(?;q?;q12) | 1x |
| **M 45** | der(2)t(2;15)(q34;q?) | 1 |
| **M 46** | der(15)ins(15;2;7)(q12;?;?)t(15;7)(q26;?) | 1x |
| **M 47** | **i(5)(p10)** | **5x** |
| **M 48** | **del(5)(q?)** | **4x** |
| **M49** | **der(12)del(12)(p12)del(12)(q22)** | **8x** |
| **M50** | **del(12)(p12)** | **8x** |

**Table S5.** Analysis of array data sets indicated genes in the aquired ellipticine resistance phenotype

| Gene Code | Gene name | Symbol | Fold Change | Significance | Chromosome Band | Function |
|---|---|---|---|---|---|---|
| NM_ 005378.4 | v-myc myelocytomatosis viral related Oncogene, neuroblastoma derived (avian) | MYCN | -0.25 | -08.00 | 2p24.1 | Member of the MYC family and encodes a protein with a basic helix-loop-helix (bHLH) domain. This protein is located in the nucleus and must dimerize with another bHLH protein in order to bind DNA. Amplification of this gene is associated with a variety of tumors, most notably neuroblastomas |
| NM_003286 | topoisomerase (DNA) I | TOP1 | -2.39 | 16 | 20q12-q13.1 | Encodes a DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription. This enzyme catalyzes the transient breaking and rejoining of a single strand of DNA which allows the strands to pass through one another, thus altering the topology of DNA. This gene is localized to chromosome 20 and has pseudogenes which reside on chromosomes 1 and 22. |
| NM_001067.2 | topoisomerase (DNA) II alpha | TOP2A | -2.00 | 17 | 17q21-q22 | This gene encodes a DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription. This nuclear enzyme is involved in processes such as chromosome condensation, chromatid separation, and the relief of torsional stress that occurs during DNA transcription and replication. It catalyzes the transient breaking and rejoining of two strands of duplex DNA which allows the strands to pass through one another, thus altering the topology of DNA. Two forms of this enzyme exist as likely products of a gene duplication event. The gene encoding this form, alpha, is localized to chromosome 17 and the beta gene is localized to chromosome 3. The gene encoding this enzyme functions as the target for several anticancer agents and a variety of mutations in this gene have been associated with the development of drug resistance. Reduced activity of this enzyme may also play a role in ataxia-telangiectasia. |
| NM_001068.2 | topoisomerase (DNA) II beta 180kDa | TOP2B | -1.29 | 11 | 3q24 | Encodes a DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription. This nuclear enzyme is involved in processes such as chromosome condensation, chromatid separation, and the relief of torsional stress that occurs during DNA transcription and replication. It catalyzes the transient breaking and rejoining of two strands of duplex DNA which allows the strands to pass through one another, thus altering the topology of DNA. Two forms of this enzyme exist as likely products of a gene duplication event. The gene encoding this form, beta, is localized to chromosome 3 and the alpha form is localized to chromosome 17. The gene encoding this enzyme functions as the target for several anticancer agents and a variety of mutations in this gene have been associated with the development of drug resistance. Reduced activity of this enzyme may also play a role in ataxia-telangiectasia. Alternative splicing of this gene results in two transcript variants; however, the second variant has not yet been fully described. |
| NM_004595.3 | spermine synthetase | SMS | +1.47 | | Xp22.1 | The protein encoded by this gene belongs to the spermidine/spermine synthases family. This gene encodes an ubiquitous enzyme of polyamine metabolism. |
| NM_003132.2 | spermidine synthase | SRM | +1.25 | | 1p36-p22 | The polyamines putrescine, spermine, and spermidine are ubiquitous polycationic |

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| | | | | | mediators of cell growth and differentiation. Spermidine synthase is one of four enzymes in the polyamine-biosynthetic pathway and carries out the final step of spermidine biosynthesis. This enzyme catalyzes the conversion of putrescine to spermidine using decarboxylated S-adenosylmethionine as the cofactor. |
| NM_000927.3 | ATP-binding cassette, sub-family B (MDR/TAP), member 1 | ABCB1 | -2.03 | 7q21.12 | The membrane-associated protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the MDR/TAP subfamily. Members of the MDR/TAP subfamily are involved in multidrug resistance. The protein encoded by this gene is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs. This protein also functions as a transporter in the blood-brain barrier. |
| NM_004996.3 | ATP-binding cassette, sub-family C (CFTR/MRP), member 1 | ABCC1 | -1.62 | 16p13.1 | The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This full transporter is a member of the MRP subfamily which is involved in multi-drug resistance. This protein functions as a multispecific organic anion transporter, with oxidized glutathione, cysteinyl leukotrienes, and activated aflatoxin B1 as substrates. This protein also transports glucuronides and sulfate conjugates of steroid hormones and bile salts. Alternative splicing by exon deletion results in several splice variants but maintains the original open reading frame in all forms. |
| NM_005688.2 | ATP-binding cassette, sub-family C (CFTR/MRP), member 5 | ABCC5 | -1.56 | 3q27 | The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the MRP subfamily which is involved in multi-drug resistance. This protein functions in the cellular export of its substrate, cyclic nucleotides. This export contributes to the degradation of phosphodiesterases and possibly an elimination pathway for cyclic nucleotides. Studies show that this protein provides resistance to thiopurine anticancer drugs, 6-mercaptopurine and thioguanine, and the anti-HIV drug 9-(2-phosphonylmethoxyethyl)adenine. This protein may be involved in resistance to thiopurines in acute lymphoblastic leukemia and antiretroviral nucleoside analogs in HIV-infected patients. Alternative splicing of this gene has been detected; however, the complete sequence and translation initiation site is unclear. |
| NM_172232.1 | ATP-binding cassette, sub-family A (ABC1), member 5 | ABCA5 | -1.55 | 17q24.3 | The membrane-associated protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intracellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, and White). This encoded protein is a member of the ABC1 subfamily. Members of the ABC1 subfamily comprise the only major ABC subfamily found |

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|---|---|---|---|---|---|
| | | | | | exclusively in multicellular eukaryotes. This gene is clustered among 4 other ABC1 family members on 17q24, but neither the substrate nor the function of this gene is known. Alternative splicing of this gene results in several transcript variants; however, not all variants have been fully described. |
| NM_003845.1 | ATP-binding cassette, sub-family C (CFTR/MRP), member 4 | ABCC4 | -1.35 | 13q32 | The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the MRP subfamily which is involved in multi-drug resistance. The specific function of this protein has not yet been determined, however, this protein may play a role in cellular detoxification as a pump for its substrate, organic anions. Alternative splicing results in multiple splice variants encoding different isoforms. |
| NM_002940.1 | ATP-binding cassette, sub-family E (OABP), member 1 | ABCE1 | -1.21 | 4q31 | The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the OABP subfamily. Alternatively referred to as the RNase L inhibitor, this protein functions to block the activity of ribonuclease L. Activation of ribonuclease L leads to inhibition of protein synthesis in the 2-5A/RNase L system, the central pathway for viral interferon action. Two transcript variants encoding the same protein have been found for this gene. |
| NM_012089.1 | ATP-binding cassette, sub-family B (MDR/TAP), member 10 | ABCB10 | -1.20 | 1q42.13 | The membrane-associated protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the MDR/TAP subfamily. Members of the MDR/TAP subfamily are involved in multidrug resistance. The function of this mitochondrial protein is unknown. |
| NM_002858.2 | ATP-binding cassette, sub-family D (ALD), member 3 | ABCD3 | -1.09 | 1p22-p21 | The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the ALD subfamily, which is involved in peroxisomal import of fatty acids and/or fatty acyl-CoAs in the organelle. All known peroxisomal ABC transporters are half transporters which require a partner half transporter molecule to form a functional homodimeric or heterodimeric transporter. This peroxisomal membrane protein likely plays an important role in peroxisome biogenesis. Mutations have been associated with some forms of Zellweger syndrome, a heterogeneous group of peroxisome assembly disorders. Alternative splicing results in multiple transcript variants encoding distinct isoforms. |
| NM_000499.2 | cytochrome P450, family 1, subfamily A, polypeptide 1 | CYP1A1 | -0.55 | 2.28 | 15q24.1 | This gene, CYP1A1, encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and its expression is |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | induced by some polycyclic aromatic hydrocarbons (PAHs), some of which are found in cigarette smoke. The enzyme's endogenous substrate is unknown; however, it is able to metabolize some PAHs to carcinogenic intermediates. The gene has been associated with lung cancer risk. A related family member, CYP1A2, is located approximately 25 kb away from CYP1A1 on chromosome 15. |
| NM_000104.2 | cytochrome P450, family 1, subfamily B, polypeptide 1 | CYP1B1 | -0.32 | -0.53 | 2q21 | This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The enzyme encoded by this gene localizes to the endoplasmic reticulum and metabolizes procarcinogens such as polycyclic aromatic hydrocarbons and 17beta-estradiol. Mutations in this gene have been associated with primary congenital glaucoma, therefore it is thought that the enzyme also metabolizes a signaling molecule involved in eye development, possibly a steroid. |
| NM_017460 | cytochrome P450, family 3, subfamily A, polypeptide 4 | CYP3A4 | +0.17 | -6.63 | 7q21.1 | This gene, CYP3A4, encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and its expression is induced by glucocorticoids and some pharmacological agents. This enzyme is involved in the metabolism of approximately half the drugs which are used today, including acetaminophen, codeine, cyclosporin A, diazepam and erythromycin. The enzyme also metabolizes some steroids and carcinogens. This gene is part of a cluster of cytochrome P450 genes on chromosome 7q21.1. Previously another CYP3A gene, CYP3A3, was thought to exist; however, it is now thought that this sequence represents a transcript variant of CYP3A4. |
| NM_000106.4 | cytochrome P450, family 2, subfamily D, polypeptide 6 | CYP2D6 | +0.30 | 2.23 | 22q13.1 | This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and is known to metabolize as many as 20% of commonly prescribed drugs. Its substrates include debrisoquine, an adrenergic-blocking drug; sparteine and propafenone, both anti-arrhythmic drugs; and amitriptyline, an anti-depressant. The gene is highly polymorphic in the population; certain alleles result in the poor metabolizer phenotype, characterized by a decreased ability to metabolize the enzyme's substrates. The gene is located near two cytochrome P450 pseudogenes on chromosome 22q13.1. Alternatively spliced transcript variants encoding different isoforms have been found for this gene. |
| NM_001914.2 | cytochrome b5 type A (microsomal) | CYB5 | +1.27 | 12.84 | 18q23 | Encodes a member of the cytochrome P450 superfamily of enzymes, monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and its expression is induced by rifampin. The enzyme is known to metabolize many xenobiotics, including phenytoin, tolbutamide, ibuprofen and S-warfarin. Studies identifying individuals who are poor metabolizers of phenytoin and tolbutamide suggest that this gene is polymorphic. The gene is located within a cluster of cytochrome P450 genes on chromosome 10q24 |
| NM_000771.3 | cytochrome P450, family 2, subfamily C, | CYP2C9 | -0.14 | -1.35 | 10q24 | This gene encodes a member of the cytochrome P450 superfamily of enzymes. The |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | polypeptide 9 | | | | | | cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and its expression is induced by rifampin. The enzyme is known to metabolize many xenobiotics, including phenytoin, tolbutamide, ibuprofen and S-warfarin. Studies identifying individuals who are poor metabolizers of phenytoin and tolbutamide suggest that this gene is polymorphic. The gene is located within a cluster of cytochrome P450 genes on chromosome 10q24. |
| | NM_000903 | NAD(P)H dehydrogenase, quinone 1 | NQO1 | -2.09 | 15.74 | 19q12 | This gene is a member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. This FAD-binding protein forms homodimers and reduces quinones to hydroquinones. This protein's enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species. Mutations in this gene have been associated with tardive dyskinesia (TD), an increased risk of hematotoxicity after exposure to benzene, and susceptibility to various forms of cancer. Altered expression of this protein has been seen in many tumors and is also associated with Alzheimer's disease (AD). Alternate transcriptional splice variants, encoding different isoforms, have been characterized. |
| | NM_000250.1 | myeloperoxidase | MPO | +0.03 | -8.38 | 17q23.1 | Myeloperoxidase (MPO) is a heme protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules. Produced as a single chain precursor, myeloperoxidase is subsequently cleaved into a light and heavy chain. The mature myeloperoxidase is a tetramer composed of 2 light chains and 2 heavy chains. This enzyme produces hypohalous acids central to the microbicidal activity of neutrophils. |
| | NM_006151.1 | lactoperoxidase | LPO | +0.05 | -6.50 | 17q23.1 | This gene encodes an oxidoreductase secreted from salivary, mammary, and other mucosal glands that functions as a natural antibacterial agent. Multiple transcript variants encoding different isoforms have been found for this gene. |
| | NM_000941.1 | P450 (cytochrome) oxidoreductase | POR | -0.06 | -5.08 | 17q11.2 | This gene encodes an endoplasmic reticulum membrane oxidoreductase with an FAD-binding domain and a flavodoxin-like domain. The protein binds two cofactors, FAD and FMN, which allow it to donate electrons directly from NADPH to all microsomal P450 enzymes. Mutations in this gene have been associated with various diseases, including apparent combined P450C17 and P450C21 deficiency, amenorrhea and disordered steroidogenesis, congenital adrenal hyperplasia and Antley-Bixler syndrome. |
| | NM_000662.4 | N-acetyltransferase 5 (GCN5-related, putative) | NAT1 | -0.90 | 5.94 | 8p23.1-p21.3 | This gene is one of two arylamine N-acetyltransferase (NAT) genes in the human genome, and is orthologous to the mouse and rat Nat2 genes. The enzyme encoded by this gene catalyzes the transfer of an acetyl group from acetyl-CoA to various aryl amine and hydrazine substrates. This enzyme helps metabolize drugs and other xenobiotics, and functions in folate catabolism. Multiple transcript variants encoding different isoforms have been found for this gene.<br>Note: Loci in other organisms that are functionally homologous to this one are validly referred to as both NAT1 and NAT2; i.e., the functional homologs of NAT1 include mouse and rat Nat2, while the functional homologs of human NAT2 include mouse and rat Nat1. Name sequence associations are consistent with current use in the field. |

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| NM_000015.1 | N-acetyltransferase 2 (arylamine N-acetyltransferase) | NAT2 | -0.03 | -7.97 | 8p22 | This gene encodes an enzyme that functions to both activate and deactivate arylamine and hydrazine drugs and carcinogens. Polymorphisms in this gene are responsible for the N-acetylation polymorphism in which human populations segregate into rapid, intermediate, and slow acetylator phenotypes. Polymorphisms in this gene are also associated with higher incidences of cancer and drug toxicity. A second arylamine N-acetyltransferase gene (NAT1) is located near this gene (NAT2). Note: Loci in other organisms that are functionally homologous to this one are validly referred to as both NAT1 and NAT2; i.e., the functional homologs of NAT1 include mouse and rat Nat2, while the functional homologs of human NAT2 include mouse and rat Nat1. Name sequence associations are consistent with current use in the field. |
| NM_016100.2 | N-acetyltransferase 5 (GCN5-related, putative) | NAT5 | -1.20 | 7.74 | 20p11.23 | NAT5 is a component of N-acetyltransferase complex B (NatB). Human NatB performs cotranslational N(alpha)-terminal acetylation of methionine residues when they are followed by asparagine. |
| NM_012191.2 | N-acetyltransferase 6 (GCN5-related) | NAT6 | +0.31 | 2.43 | 3p21.3 | Seems to be involved in N-acetylation. Acts on peptides with a N-terminal Met followed by Asp/Glu/Asn. May act as a tumor suppressor |
| NM_003960 | N-acetyltransferase 8 (GCN5-related, putative) | NAT8 | +0.03 | -8.45 | 2p13.1-p12 | This gene, isolated using the differential display method to detect tissue-specific genes, is specifically expressed in kidney and liver. The encoded protein shows amino acid sequence similarity to N-acetyltransferases. A similar protein in Xenopus affects cell adhesion and gastrulation movements, and may be localized in the secretory pathway. A highly similar paralog is found in a cluster with this gene. |
| NM_015654.3 | N-acetyltransferase 9 (GCN5-related, putative) | NAT9 | -0.19 | 2.19 | 17q25.1 | Belongs to the acetyltransferase family. GNAT subfamily. Contains 1 N-acetyltransferase domain. |
| NM_138761.2 | BCL2-associated X protein | BAX | +0.86 | 10.90 | 19q13.3-q13.4 | The protein encoded by this gene belongs to the BCL2 protein family. BCL2 family members form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. This protein forms a heterodimer with BCL2, and functions as an apoptotic activator. This protein is reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome c. The expression of this gene is regulated by the tumor suppressor P53 and has been shown to be involved in P53-mediated apoptosis. Multiple alternatively spliced transcript variants, which encode different isoforms, have been reported for this gene. |
| NM_000657.1 | B-cell CLL/lymphoma 2 | BCL-2 | 0.78 | 8.60 | 18q21.3 | This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes. Constitutive expression of BCL2, such as in the case of translocation of BCL2 to Ig heavy chain locus, is thought to be the cause of follicular lymphoma. Two transcript variants, produced by alternate splicing, differ in their C-terminal ends. |
| NM_177536.1 | sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 | SULT1A1 | -0.37 | 5.24 | 16p12.1 | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. This gene encodes |

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| | | | | | | | one of two phenol sulfotransferases with thermostable enzyme activity. Multiple alternatively spliced variants that encode two isoforms have been identified for this gene. |
| NM_177528.1 | sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2 | SULT1A2 | -0.19 | -2.99 | 16p12.1 | | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. This gene encodes one of two phenol sulfotransferases with thermostable enzyme activity. Two alternatively spliced variants that encode the same protein have been described. |
| NM_001017387.1 | sulfotransferase family, cytosolic, 1A, phenol -preferring, member 3 | SULT1A3 | -0.21 | 1.96 | 6p11.2 | | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. This gene encodes a phenol sulfotransferase with thermolabile enzyme activity. Four sulfotransferase genes are located on the p arm of chromosome 16; this gene and SULT1A4 arose from a segmental duplication. This gene is the most centromeric of the four sulfotransferase genes. Exons of this gene overlap with exons of a gene that encodes a protein containing GIY-YIG domains (GIYD1). Multiple alternatively spliced variants that encode the same protein have been described. |
| - | sulfotransferase family, cytosolic, 1B, member 1 | SULT1B1 | -0.12 | -5.73 | 4q13.3 | | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. However, the total genomic length of this gene is greater than that of other SULT1 genes. |
| NM_176825.2 | sulfotransferase family, cytosolic, 1C, member 1 | SULT1C1 | -0.26 | 0.25 | 2q11.1-q11.2 | | |
| NM_006588 | sulfotransferase family, cytosolic, 1C, member 2 | SULT1C2 | -0.42 | 3.57 | 2q11.1-q11.2 | | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. This gene encodes a protein that belongs to the SULT1 subfamily, responsible for transferring a sulfo moiety from PAPS to phenol-containing compounds. Two alternatively spliced transcript variants encoding different isoforms have been described for this gene. |
| NM_005420.2 | sulfotransferase family 1E, estrogen-preferring, member 1 | SULT1E1 | +0.46 | -3.51 | 4q13.1 | | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. This gene encodes a protein that transfers a sulfo moiety to and from estrone, which may control levels of estrogen receptors. |
| NM_176874.1 | sulfotransferase family 4A, member 1 | SULT4A1 | +0.35 | 2.60 | 22q13.2-q13.31 | | This gene encodes a member of the sulfotransferase family. The encoded protein is a |

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| | | | | | | | brain-specific sulfotransferase believed to be involved in the metabolism of neurotransmitters. Polymorphisms in this gene may be associated with susceptibility to schizophrenia. |
| NM_004605.2 | sulfotransferase family, cytosolic, 2B, member 1 | SULT2B1 | +0.15 | 0.06 | 19q13.3 | | Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. This gene sulfates dehydroepiandrosterone but not 4-nitrophenol, a typical substrate for the phenol and estrogen sulfotransferase subfamilies. Two alternatively spliced variants that encode different isoforms have been described. |
| NM_198066.2 | glucosamine-phosphate N-acetyltransferase 1 | GNPNAT1 | +0.31 | 4.62 | 14q22.1 | | Belongs to the acetyltransferase family. GNA1 subfamily. |
| NM_000962.2 | prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | PTGS1 (COX1) | +0.13 | -3.29 | 9q32-q33.3 | | Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. There are two isozymes of PTGS: a constitutive PTGS1 and an inducible PTGS2, which differ in their regulation of expression and tissue distribution. This gene encodes PTGS1, which regulates angiogenesis in endothelial cells, and is inhibited by nonsteroidal anti-inflammatory drugs such as aspirin. PTGS1 is thought to be involved in cell-cell signaling and maintaining tissue homeostasis. Alternative splicing of this gene generates two transcript variants. The expression of these two transcripts is differentially regulated by relevant cytokines and growth factors. |
| NM_000963.1 | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | PTGS2 (COX2) | -0.21 | -0.35 | 1q25.2-q25.3 | | Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. There are two isozymes of PTGS: a constitutive PTGS1 and an inducible PTGS2, which differ in their regulation of expression and tissue distribution. This gene encodes the inducible isozyme. It is regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis. |
| NM_001088.1 | arylalkylamine N-acetyltransferase | AANAT | +0.32 | -0.07 | 17q25 | | The protein encoded by this gene belongs to the acetyltransferase superfamily. It is the penultimate enzyme in melatonin synthesis and controls the night/day rhythm in melatonin production in the vertebrate pineal gland. Melatonin is essential for the function of the circadian clock that influences activity and sleep. This enzyme is regulated by cAMP-dependent phosphorylation that promotes its interaction with 14-3-3 proteins and thus protects the enzyme against proteasomal degradation. This gene may contribute to numerous genetic diseases such as delayed sleep phase syndrome. Alternatively spliced transcript variants encoding different isoforms have been found for this gene. |
| NM_000107.1 | damage-specific DNA binding protein 2 | DDB2 | +1.09 | 12.68 | 11p12-p11 | | This gene encodes a protein that is necessary for the repair of ultraviolet light-damaged DNA. This protein is the smaller subunit of a heterodimeric protein complex that participates in nucleotide excision repair, and this complex mediates the ubiquitylation of histones H3 and H4, which facilitates the cellular response to DNA |

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| NM_002528.4 | nth endonuclease III-like 1 (E. coli) | NTHL1 | +1.38 | 13.68 | 16p13.3 | damage. This subunit appears to be required for DNA binding. Mutations in this gene cause xeroderma pigmentosum complementation group E, a recessive disease that is characterized by an increased sensitivity to UV light and a high predisposition for skin cancer development, in some cases accompanied by neurological abnormalities. |
| NM_006230.1 | polymerase (DNA directed), delta 2, regulatory subunit 50kDa | POLD2 | +1.10 | 13.08 | 7p13 | The protein encoded by this gene is a DNA N-glycosylase of the endonuclease III family. Like a similar protein in E. coli, the encoded protein has DNA glycosylase activity on DNA substrates containing oxidized pyrimidine residues and has apurinic/apyrimidinic lyase activity. |
| NM_005053.2 | RAD23 homolog A (S. cerevisiae) | RAD23A | +1.25 | 13.01 | 19p13.2 | The DNA polymerase delta complex is involved in DNA replication and repair, and it consists of the proliferating cell nuclear antigen (PCNA; MIM 176740), the multisubunit replication factor C (see MIM 102579), and the 4 subunit polymerase complex: POLD1 (MIM 174761), POLD2, POLD3 (MIM 611415), and POLD4 (MIM 611525) (Liu and Warbrick, 2006 [PubMed 16934752]). |
| NM_002592.2 | proliferating cell nuclear antigen | PCNA | +1.04 | 9.68 | 20pter-p12 | The protein encoded by this gene is one of two human homologs of Saccharomyces cerevisiae Rad23, a protein involved in nucleotide excision repair (NER). This protein was shown to interact with, and elevate the nucleotide excision activity of 3-methyladenine-DNA glycosylase (MPG), which suggested a role in DNA damage recognition in base excision repair. This protein contains an N-terminal ubiquitin-like domain, which was reported to interact with 26S proteasome, as well as with ubiquitin protein ligase E6AP, and thus suggests that this protein may be involved in the ubiquitin mediated proteolytic pathway in cells. |
| NM_000380.2 | xeroderma pigmentosum | XPA | +0.93 | 9.42 | 9q22.3 | The protein encoded by this gene is found in the nucleus and is a cofactor of DNA polymerase delta. The encoded protein acts as a homotrimer and helps increase the processivity of leading strand synthesis during DNA replication. In response to DNA damage, this protein is ubiquitinated and is involved in the RAD6-dependent DNA repair pathway. Two transcript variants encoding the same protein have been found for this gene. Pseudogenes of this gene have been described on chromosome 4 and on the X chromosome. |
| NM_000059.1 | breast cancer 2, early onset | BRCA2 | -0.78 | 11.11 | 13q12.3 | This gene encodes a zinc finger protein involved in DNA excision repair. The encoded protein is part of the NER (nucleotide excision repair) complext which is responsible for repair of UV radiation-induced photoproducts and DNA adducts induced by chemical carcinogens. Mutations in this gene are associated with xeroderma pigmentosum complementation group A. Alternatively spliced transcript variants have been found for this gene. |
| | | | | | | Inherited mutations in BRCA1 and this gene, BRCA2, confer increased lifetime risk of developing breast or ovarian cancer. Both BRCA1 and BRCA2 are involved in maintenance of genome stability, specifically the homologous recombination pathway for double-strand DNA repair. The BRCA2 protein contains several copies of a 70 aa motif called the BRC motif, and these motifs mediate binding to the RAD51 recombinase which functions in DNA repair. BRCA2 is considered a tumor suppressor gene, as tumors with BRCA2 mutations generally exhibit loss of heterozygosity (LOH) of the wild-type allele. |

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| NM_000057.1 | Bloom syndrome, RecQ helikase-like | BLM | -1.52 | 14.21 | 15q26.1 | The Bloom syndrome gene product is related to the RecQ subset of DExH box-containing DNA helicases and has both DNA-stimulated ATPase and ATP-dependent DNA helicase activities. Mutations causing Bloom syndrome delete or alter helicase motifs and may disable the 3'-5' helicase activity. The normal protein may act to suppress inappropriate recombination. |
| NM_206937.1 | ligase IV, DNA, ATP-dependent | LIG4 | -1.16 | 13.09 | 13q33-q34 | The protein encoded by this gene is a DNA ligase that joins single-strand breaks in a double-stranded polydeoxynucleotide in an ATP-dependent reaction. This protein is essential for V(D)J recombination and DNA double-strand break (DSB) repair through nonhomologous end joining (NHEJ). This protein forms a complex with the X-ray repair cross complementing protein 4 (XRCC4), and further interacts with the DNA-dependent protein kinase (DNA-PK). Both XRCC4 and DNA-PK are known to be required for NHEJ. The crystal structure of the complex formed by this protein and XRCC4 has been resolved. Defects in this gene are the cause of LIG4 syndrome. Alternatively spliced transcript variants encoding the same protein have been observed. |
| NM_000251.1 | mutS homolog 2, nonpolyposis type 1 (E. coli) | MSH2 | -1.76 | 13.24 | 2p22-p21 | MSH2 was identified as a locus frequently mutated in hereditary nonpolyposis colon cancer (HNPCC). When cloned, it was discovered to be a human homolog of the E. coli mismatch repair gene mutS, consistent with the characteristic alterations in microsatellite sequences (RER+ phenotype) found in HNPCC. |
| NM_001024688.1 | nibrin | NBN | -1.69 | 15.10 | 8q21 | Mutations in this gene are associated with Nijmegen breakage syndrome, an autosomal recessive chromosomal instability syndrome characterized by microcephaly, growth retardation, immunodeficiency, and cancer predisposition. The encoded protein is a member of the MRE11/RAD50 double-strand break repair complex which consists of 5 proteins. This gene product is thought to be involved in DNA double-strand break repair and DNA damage-induced checkpoint activation. |
| NM_006999.3 | polymerase (DNA directed) sigma | POLS | -1.06 | 12.58 | 5p15 | The protein encoded by this gene is a DNA polymerase that is likely involved in DNA repair. In addition, the encoded protein may be required for sister chromatid adhesion. |
| NM_006904.6 | protein kinase, DNA-activated, catalytic polypeptide | PRKDC | -1.73 | 14.08 | 8q11 | The PRKDC gene encodes the catalytic subunit of a nuclear DNA-dependent serine/threonine protein kinase (DNA-PK). The second component is the autoimmune antigen Ku (MIM 152690), which is encoded by the G22P1 gene on chromosome 22q. On its own, the catalytic subunit of DNA-PK is inactive and relies on the G22P1 component to direct it to the DNA and trigger its kinase activity; PRKDC must be bound to DNA to express its catalytic properties. |
| NM_006265 | RAD21 homolog (S. pombe) | RAD21 | -2.34 | 15.05 | 8q24 | The protein encoded by this gene is highly similar to the gene product of Schizosaccharomyces pombe rad21, a gene involved in the repair of DNA double-strand breaks, as well as in chromatid cohesion during mitosis. This protein is a nuclear phospho-protein, which becomes hyperphosphorylated in cell cycle M phase. The highly regulated association of this protein with mitotic chromatin specifically at the centromere region suggests its role in sister chromatid cohesion in mitotic cells. |
| NM_005732.2 | RAD50 homolog (S. cerevisiae) | RAD50 | -1.2 | 13.72 | 5q31 | The protein encoded by this gene is highly similar to Saccharomyces cerevisiae |

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| | | | | | | Rad50, a protein involved in DNA double-strand break repair. This protein forms a complex with MRE11 and NBS1. The protein complex binds to DNA and displays numerous enzymatic activities that are required for nonhomologous joining of DNA ends. This protein, cooperating with its partners, is important for DNA double-strand break repair, cell cycle checkpoint activation, telomere maintenance, and meiotic recombination. Knockout studies of the mouse homolog suggest this gene is essential for cell growth and viability. Two alternatively spliced transcript variants of this gene, which encode distinct proteins, have been reported. |
| NM_134424.1 | RAD 52 homolog | RAD52 | -0.71 | 9.89 | 12p13-p12.2 | The protein encoded by this gene shares similarity with Saccharomyces cerevisiae Rad52, a protein important for DNA double-strand break repair and homologous recombination. This gene product was shown to bind single-stranded DNA ends, and mediate the DNA-DNA interaction necessary for the annealing of complementary DNA strands. It was also found to interact with DNA recombination protein RAD51, which suggested its role in RAD51 related DNA recombination and repair. |
| NM_012415.2 | RAD54 homolog B (S. cerevisiae) | RAD54B | -2.09 | 15.91 | 5p15 | The protein encoded by this gene belongs to the DEAD-like helicase superfamily. It shares similarity with Saccharomyces cerevisiae RAD54 and RDH54, both of which are involved in homologous recombination and repair of DNA. This protein binds to double-stranded DNA, and displays ATPase activity in the presence of DNA. This gene is highly expressed in testis and spleen, which suggests active roles in meiotic and mitotic recombination. Homozygous mutations of this gene were observed in primary lymphoma and colon cancer. |
| NM_005431 | X-ray repair complementing defective repair in Chinese hamster cells 2 | XRCC2 | -0.85 | 09.20 | 7q36.1 | This gene encodes a member of the RecA/Rad51-related protein family that participates in homologous recombination to maintain chromosome stability and repair DNA damage. This gene is involved in the repair of DNA double-strand breaks by homologous recombination and it functionally complements Chinese hamster irs1, a repair-deficient mutant that exhibits hypersensitivity to a number of different DNA-damaging agents. |
| NM_003348 | ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast) | UBE2N | -0.72 | 10.67 | 12q22 | The modification of proteins with ubiquitin is an important cellular mechanism for targeting abnormal or short-lived proteins for degradation. Ubiquitination involves at least three classes of enzymes: ubiquitin-activating enzymes, or E1s, ubiquitin-conjugating enzymes, or E2s, and ubiquitin-protein ligases, or E3s. This gene encodes a member of the E2 ubiquitin-conjugating enzyme family. Studies in mouse suggest that this protein plays a role in DNA postreplication repair. |
| NM_001469 | X-ray repair complementing defective repair in Chinese hamster cells 6 | XRCC6 | -0.69 | 10.55 | 22q13.2-q13.31 | The p70/p80 autoantigen is a nuclear complex consisting of two subunits with molecular masses of approximately 70 and 80 kDa. The complex functions as a single-stranded DNA-dependent ATP-dependent helicase. The complex may be involved in the repair of nonhomologous DNA ends such as that required for double-strand break repair, transposition, and V(D)J recombination. High levels of autoantibodies to p70 and p80 have been found in some patients with systemic lupus erythematosus. |

**TABLE S6.** Analysis of array data sets indicated predominantly statistically high significant (≥ 10) upregulated genes in the oxidative phosphorylation

| Gene Code | Gene name | Symbol | Fold Change | Significance | Chromosome band | Function |
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| NM_0010019751 | ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit | ATP5D | +1.32 | 11.40 | 19p13.3 | This gene encodes a subunit of mitochondrial ATP synthase. Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. ATP synthase is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, Fo, comprising the proton channel. The catalytic portion of mitochondrial ATP synthase consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled with a stoichiometry of 3 alpha, 3 beta, and a single representative of the other 3. The proton channel consists of three main subunits (a, b, c). This gene encodes the delta subunit of the catalytic core. Alternatively spliced transcript variants encoding the same isoform have been identified. |
| NM_006886.2 | ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit | ATP5E | +0.98 | 12.03 | 20q13.32 | This gene encodes a subunit of mitochondrial ATP synthase. Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. ATP synthase is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, Fo, comprising the proton channel. The catalytic portion of mitochondrial ATP synthase consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled with a stoichiometry of 3 alpha, 3 beta, and a single representative of the other 3. The proton channel consists of three main subunits (a, b, c). This gene encodes the epsilon subunit of the catalytic core. Two pseudogenes of this gene are located on chromosomes 4 and 13. |
| NM_005175.2 | ATP synthase, H+ transporting mitochondrial F0 complex | ATP5G1 | +1.4 | 13.45 | 17q21.32 | This gene encodes a subunit of mitochondrial ATP synthase. Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. ATP synthase is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, Fo, comprising the proton channel. The catalytic portion of mitochondrial ATP synthase consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled with a stoichiometry of 3 alpha, 3 beta, and a single representative of the other 3. The proton channel seems to have nine subunits (a, b, c, d, e, f, g, F6 and 8). This gene is one of three genes that encode subunit c of the proton channel. Each of the three genes have distinct mitochondrial import sequences but encode the identical mature protein. Alternatively spliced transcript variants encoding the same protein have been identified. |
| NM_0010037851 | ATP synthase, H+ transporting | ATP5H | +0.74 | 10.62 | 17q25 | Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical |

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| Mitochondrial F0 complex, subunit d | | | | | | gradient of protons across the inner membrane during oxidative phosphorylation. It is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, F0, which comprises the proton channel. The F1 complex consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled in a ratio of 3 alpha, 3 beta, and a single representative of the other 3. The F0 seems to have nine subunits (a, b, c, d, e, f, g, F6 and 8). This gene encodes the d subunit of the F0 complex. Alternatively spliced transcript variants encoding different isoforms have been identified for this gene. In addition, three pseudogenes are located on chromosomes 9, 12 and 15. |
| NM_007100.2 | ATP synthase, H+ transporting mitochondrial F0 complex, subunit E | ATP5I | +0.84 | 11.38 | 4p16.3 | Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. It is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, F0, which comprises the proton channel. The F1 complex consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled in a ratio of 3 alpha, 3 beta, and a single representative of the other 3. The F0 seems to have nine subunits (a, b, c, d, e, f, g, F6 and 8). This gene encodes the e subunit of the F0 complex. |
| NM_001685 | ATP synthase, H+ transporting mitochondrial F0 complex, subunit F6 | ATP5J | +0.96 | 11.69 | 21q21.1 | Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. It is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, F0, which comprises the proton channel. The F1 complex consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled in a ratio of 3 alpha, 3 beta, and a single representative of the other 3. The F0 seems to have nine subunits (a, b, c, d, e, f, g, F6 and 8). This gene encodes the F6 subunit of the F0 complex, required for F1 and F0 interactions. Alternatively spliced transcript variants encoding different isoforms have been identified for this gene. |
| NM_006476.4 | ATP synthase, H+ transporting mitochondrial F0 complex, subunit G | ATP5L | +0.91 | 11.20 | 11q23.3 | Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. It is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, F0, which comprises the proton channel. The F1 complex consists of 5 different subunits (alpha, beta, gamma, delta, and epsilon) assembled in a ratio of 3 alpha, 3 beta, and a single representative of the other 3. The F0 seems to have nine subunits (a, b, c, d, e, f, g, F6 and 8). This gene encodes the g subunit of the F0 complex. |
| NM_001697.2 | ATP synthase, H+ transporting | ATP5O | +0.76 | 10.77 | 21q22.1-q22.2; 21q22.11 | The protein encoded by this gene is a component of the F-type ATPase found in the mitochondrial matrix. F-type ATPases are composed of a catalytic core and a membrane proton channel. The encoded protein appears to be part of the connector linking these two components and may be involved in transmission of conformational changes or proton conductance. |
| NM_001183.3 | ATPase, H+ transporting lysosomal accessory protein 1 | ATP6AP1 | +0.80 | 10.63 | Xq28 | This gene encodes a component of a multisubunit enzyme (1 mDa MW) that mediates acidification of eukaryotic intracellular organelles. Vacuolar ATPase (V-ATPase) is comprised of a cytosolic V1 (site of the ATP catalytic site) and a transmembrane V0 domain. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, and receptor- |

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| | | | | | | mediated endocytosis. The encoded protein of this gene is approximately 45 kD and may assist in the V-ATPase-mediated acidification of neuroendocrine secretory granules. |
| NM_004691.3 | ATPase, H+ transporting lysosomal 38kDa, V0 subunit d1 | ATP6V0D1 | +1.1 | 16q22 | | This gene encodes a component of vacuolar ATPase (V-ATPase), a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation. V-ATPase is composed of a cytosolic V1 domain and a transmembrane V0 domain. The V1 domain consists of three A and three B subunits, two G subunits plus the C, D, E, F, and H subunits. The V1 domain contains the ATP catalytic site. The V0 domain consists of five different subunits: a, c, c', c", and d. Additional isoforms of many of the V1 and V0 subunit proteins are encoded by multiple genes or alternatively spliced transcript variants. This encoded protein is known as the D subunit and is found ubiquitously. |
| NM_003945.3 | ATPase, H+ transporting lysosomal 9kDa, V0 subunit e1 | ATP6V0E1 | +1.11 | 11.81 | 5q35.2 | This gene encodes a component of vacuolar ATPase (V-ATPase), a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation. V-ATPase is composed of a cytosolic V1 domain and a transmembrane V0 domain. The V1 domain consists of three A and three B subunits, two G subunits plus the C, D, E, F, and H subunits. The V1 domain contains the ATP catalytic site. The V0 domain consists of five different subunits: a, c, c', c", and d. Additional isoforms of many of the V1 and V0 subunit proteins are encoded by multiple genes or alternatively spliced transcript variants. This encoded protein is possibly part of the V0 subunit. Since two nontranscribed pseudogenes have been found in dog, it is possible that the localization to chromosome 2 for this gene by radiation hybrid mapping is representing a pseudogene. Genomic mapping puts the chromosomal location on 5q35.3. |
| NM_004231 | ATPase, H+ transporting | ATP6V1F | +1.06 | 11.2 | 7q32 | This gene encodes a component of vacuolar ATPase (V-ATPase), a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, receptor-mediated endocytosis, and synaptic vesicle proton gradient generation. V-ATPase is composed of a cytosolic V1 domain and a transmembrane V0 domain. The V1 domain consists of three A and three B subunits, two G subunits plus the C, D, E, F, and H subunits. The V1 domain contains the ATP catalytic site. The V0 domain consists of five different subunits: a, c, c', c", and d. Additional isoforms of many of the V1 and V0 subunit proteins are encoded by multiple genes or alternatively spliced transcript variants. This encoded protein is the V1 domain F subunit protein. |
| NM_004541.2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1 | NDUFA1 | +0.99 | 12.32 | Xq24 | The human NDUFA1 gene codes for an essential component of complex I of the respiratory chain, which transfers electrons from NADH to ubiquinone. It has been noted that the N-terminal hydrophobic domain has the potential to be folded into an alpha-helix spanning the inner mitochondrial membrane with a C-terminal hydrophilic domain interacting with globular subunits of complex I. The highly |

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| | | | | | | conserved two-domain structure suggests that this feature is critical for the protein function and might act as an anchor for the NADH ubiquinone oxidoreductase complex at the inner mitochondrial membrane. However, the NDUFA1 peptide is one of about 31 components of the "hydrophobic protein" (HP) fraction of complex I which is involved in proton translocation. Thus the NDUFA1 peptide may also participate in that function. |
| NM_002488.2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2 | NDUFA2 +1.13 | 13.17 | 5q31 | | The NDUFA2 gene encodes one of the accessory subunits of complex I, the first and largest complex of the mitochondrial respiratory chain. |
| NM_003000.2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 | NDUFA5 +0.83 | 10.16 | 7q32 | | The human NDUFA5 gene codes for the B13 subunit of complex I of the respiratory chain, which transfers electrons from NADH to ubiquinone. The high degree of conservation of NDUFA5 extending to plants and fungi indicates its functional significance in the enzyme complex. The protein localizes to the inner mitochondrial membrane as part of the 7 component-containing, water soluble "iron-sulfur protein" (IP) fraction of complex I, although its specific role is unknown. It is assumed to undergo post-translational removal of the initiator methionine and N-acetylation of the next amino acid. The predicted secondary structure is primarily alpha helix, but the carboxy-terminal half of the protein has high potential to adopt a coiled-coil form. The amino-terminal part contains a putative beta sheet rich in hydrophobic amino acids that may serve as mitochondrial import signal. Related pseudogenes have also been identified on four other chromosomes. |
| NM_014222.2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex | NDUFA8 +1.31 | 13.99 | 9q33.2-q34.11 | | The protein encoded by this gene belongs to the complex I 19 kDA subunit family. Mammalian complex I is composed of 45 different subunits. This protein has NADH dehydrogenase activity and oxidoreductase activity. It plays an important role in transferring electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. |
| NM_002491 | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3 | NDUFB3 +0.94 | 10.71 | 2q31.3 | | The multisubunit NADH ubiquinone oxidoreductase (complex I) is the first enzyme complex in the electron transport chain of mitochondria. |
| NM_004547 | NADH dehydrogenase (ubiquinone) 1 beta subcomplex | NDUFB4 +1.33 | 13.46 | 3q13.33 | | The protein encoded by this gene belongs to the complex I NDUFB4 subunit family. Mammalian complex I is composed of 45 different subunits. This protein has NADH dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. |
| NM_002492.2 | NADH dehydrogenase (ubiquinone) | NDUFB5 +0.95 | 12.26 | 3q26.33 | | The protein encoded by this gene is a subunit of the multisubunit NADH ubiquinone oxidoreductase (complex I). Mammalian complex I is composed of 45 different subunits. It locates at the mitochondrial inner membrane. This protein has NADH dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. |
| NM_004146.4 | NADH dehydrogenase (ubiquinone) 1 | NDUFB7 +1.22 | 12.40 | 19p13.12-p13.11 | | The protein encoded by this gene is a subunit of the multisubunit NADH ubiquinone oxidoreductase (complex I). Mammalian complex I is composed of 45 different subunits. It is located at the mitochondrial inner membrane. This protein has NADH |

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| | | | | | | | dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. |
| NM_021075.3 | NADH dehydrogenase (ubiquinone) | NDUFV3 | +0.81 | 11.04 | 21q22.3 | | The protein encoded by this gene is one of at least forty-one subunits that make up the NADH-ubiquinone oxidoreductase complex. This complex is part of the mitochondrial respiratory chain and serves to catalyze the rotenone-sensitive oxidation of NADH and the reduction of ubiquinone. The encoded protein is one of three proteins found in the flavoprotein fraction of the complex. The specific function of the encoded protein is unknown. Two transcript variants encoding different isoforms have been found for this gene. |
| NM_004551.1 | NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase) | NDUFS3 | +1.44 | 10.78 | 11p11.11 | | This gene encodes one of the iron-sulfur protein (IP) components of mitochondrial NADH ubiquinone oxidoreductase (complex I). Mutations in this gene are associated with Leigh syndrome resulting from mitochondrial complex I deficiency |
| NM_002495.1 | NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase) | NDUFS4 | +1.24 | 12.17 | 5q11.1 | | This gene encodes an accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), or NADH:ubiquinone oxidoreductase, the first multi-subunit enzyme complex of the mitochondrial respiratory chain. Complex I plays a vital role in cellular ATP production, the primary source of energy for many crucial processes in living cells. It removes electrons from NADH and passes them by a series of different protein-coupled redox centers to the electron acceptor ubiquinone. In well-coupled mitochondria, the electron flux leads to ATP generation via the building of a proton gradient across the inner membrane. Complex I is composed of at least 41 subunits, of which 7 are encoded by the mitochondrial genome and the remainder by nuclear genes. |
| NM_001003684.1 | ubiquinol-cytochrome c reductase complex | UCRC | +1.38 | 13.69 | 22cen-q12.3 | | UCRC is a subunit of mitochondrial complex III (ubiquinol-cytochrome c reductase; EC 1.10.2.2), which forms the middle segment of the respiratory chain of the inner mitochondrial membrane. |

## Práce IV: Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells

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interdisciplinary toxicology

**ORIGINAL ARTICLE**

# Histone deacetylase inhibitors valproate and trichostatin A are toxic to neuroblastoma cells and modulate cytochrome P450 1A1, 1B1 and 3A4 expression in these cells

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**ABSTRACT**

Histone deacetylase inhibitors such as valproic acid (VPA) and trichostatin A (TSA) were shown to exert antitumor activity. Here, the toxicity of both drugs to human neuroblastoma cell lines was investigated using MTT test, and IC50 values for both compounds were determined. Another target of this work was to evaluate the effects of both drugs on expression of cytochrome P450 (CYP) 1A1, 1B1 and 3A4 enzymes, which are known to be expressed in neuroblastoma cells. A malignant subset of neuroblastoma cells, so-called N-type cells (UKF-NB-3 cells) and the more benign S-type neuroblastoma cells (UKF-NB-4 and SK-N-AS cell lines) were studied from both two points of view. VPA and TSA inhibited the growth of neuroblastoma cells in a dose-dependent manner. The $IC_{50}$ values ranging from 1.0 to 2.8 mM and from 69.8 to 129.4 nM were found for VPA and TSA, respectively. Of the neuroblastoma tested here, the N-type UKF-NB-3 cell line was the most sensitive to both drugs. The different effects of VPA and TSA were found on expression of CYP1A1, 1B1 and 3A4 enzymes in individual neuroblastoma cells tested in the study. Protein expression of all these CYP enzymes in the S-type SK-N-AS cell line was not influenced by either of studied drugs. On the contrary, in another S-type cell line, UKF-NB-4, VPA and TSA induced expression of CYP1A1, depressed levels of CYP1B1 and had no effect on expression levels of CYP3A4 enzyme. In the N-type UKF-NB-3 cell line, the expression of CYP1A1 was strongly induced, while that of CYP1B1 depressed by VPA and TSA. VPA also induced the expression of CYP3A4 in this neuroblastoma cell line.

**KEY WORDS:** histone deacetylase inhibitors; valproate; trichostatin A; neuroblastoma; cytotoxicity

## Introduction

Neuroblastoma is the major cause of death from neoplasia in infancy (Maris and Mathay, 1999). These solid extracranial tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential (Brodeur, 2003). Low-risk neuroblastoma is one of the rare human malignancies that are known to demonstrate spontaneous regression in infants from an undifferentiated state to a completely benign cellular formation (ganglioneuroma), whereas high-risk neuroblastoma grows relentlessly and may be rapidly fatal. Prognosis of high-risk form of cancer is poor, because drug resistance arises in the majority of those patients, initially responding to chemotherapy (Brodeur, 2003).

Neuroblastoma consists of two principal neoplastic cells (Voigt *et al.*, 2000; Hopkins-Donaldson *et al.*, 2004): i) neuroblastic or N-type: undifferentiated, round and small scant cytoplasm cells; and ii) stromal or S-type: large hyaline, flattened and adherent differentiated cells. As neuroblastoma cells seem to have the capacity to differentiate spontaneously *in vivo* and *in vitro* (Morgenstern *et al.*, 2004), their heterogeneity could affect treatment outcome, in particular the response to apoptosis induced by chemotherapy.

To achieve the most suitable concept of treatment, drugs are usually used in various combinations. Agents commonly used in neuroblastoma treatment are platinum compounds (cisplatin, carboplatin), alkylating agents (cyclophosphamide, ifosfamide, melphalan), topoisomerase II inhibitors (etoposide), anthracycline antibiotics (doxorubicin) and

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vinca alkaloids (vincristine). Some novel regimen include also topoisomerase I inhibitors (topotecan and irinotecan) that are effective against recurrent disease (Brodeur, 2003).

Because the epigenetic structure of DNA and its lesions play a role in the origin of human neuroblastomas, pharmaceutical manipulation of the epigenome may offer other treatment options also for neuroblastomas (Furchert *et al.*, 2007). Histone deacetylases (HDAC) and histone acetyl transferases modify histone proteins and contribute to an epigenetic code recognized by proteins involved in regulation of gene expression (Marks *et al.*, 2003; 2004; Hooven *et al.*, 2005). Indeed, former studies demonstrated the cytotoxicity of HDAC inhibitors to several neuroblastoma cells, resulting in growth inhibition of these tumor cells (Cinatl *et al.*, 1996; Michaelis *et al.*, 2004; 2007; Furchert *et al.*, 2007). In neoplastic cells, where overexpression of different HDACs was frequently detected (for summary see, Bolden *et al.*, 2006), the abundance of deacetylated histones is usually associated with DNA hypermethylation and gene silencing (Santini *et al.*, 2007). Treatment with HDAC inhibitors induced the reactivation of growth regulatory genes and consequently apoptosis in these cells. One of the HDAC inhibitors, valproic acid (VPA), inhibits growth and induces differentiation of human neuroblastoma UKF-NB-2 and UKF-NB-3 cells *in vitro* at concentrations ranging from 0.5 to 2 mM that have been achieved in human with no significant adverse effects (Cinatl *et al.*, 1996). However, information on effects of VPA and other HDAC inhibitors on additional neuroblastoma cells are scarce. Therefore, here we extended this study by investigating the effect of VPA and another HDAC inhibitor, trichostatin A (TSA), on other neuroblastoma cell lines. Because heterogeneity of neuroblastoma cells could affect their treatment, two types of neuroblastoma cell lines were tested for their response to VPA and TSA treatment. Besides the effect of VPA and TSA on UKF-NB-3 cells (the invasive N-type), that on the UKF-NB-4 and SK-N-AS cell lines (the non-invasive and less-aggressive S-type) was investigated in this work.

In addition, VPA and TSA are known to be metabolized by cytochrome P450 (CYP) biotransformation enzymes and can increase and/or decrease their activities and/or expression, thereby affecting mechanisms that control drug disposition (Fisher *et al.*, 1991; Rogiers *et al.*, 1992; 1995; Isojärvi *et al.*, 2001; Wen *et al.*, 2001; Bort *et al.*, 2004; Cerveny *et al.*, 2007; Nelson-DeGrave *et al.*, 2004; Hooven *et al.*, 2005; Snykers *et al.*, 2007; Kiang *et al.*, 2006). Because several CYP enzymes metabolizing a variety of drugs (CYP1A1, 1B1 and 3A4) were found to be expressed in neuroblastoma cells (Poljaková *et al.*, 2009), here we also investigated whether their expression is influenced by VPA and TSA in these cells.

## Material and methods

### Chemicals

Valproate and trichostatin A were obtained from Sigma (St. Louis, MO, USA). All other chemicals used in the experiments were of analytical purity or better.

### Cell cultures

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high-risk neuroblastoma, were a gift of prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). Cell line UKF-NB-4 was established from infiltrated bone marrow of chemoresistant high-risk neuroblastoma recurrence and have high expression of P-glycoprotein. SK-N-AS, derived from bone marrow metastases of neuroblastoma, was of the commercial source (ECACC, Salisbury, UK). Cells were grown at 37 °C and 5% $CO_2$ in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 µg/ml streptomycine (PAA Laboratories, Pasching, Austria).

### MTT assay

The cytotoxicity of valproate and trichostatin A was determined by MTT test. For a dose-response curve, culture medium stock solutions of valproate (200 mM) and DMSO solutions of trichostatin A (1 mM) were dissolved in culture medium to final concentrations of 0 – 50 mM and 0 – 1 µM for valproate and trichostatin A, respectively. Cells in exponential growth were seeded at $1 \times 10^4$ per well in a 96-well microplate. After incubation (72 hours) at 37 °C in 5% $CO_2$ saturated atmosphere the MTT solution (2 mg/ml PBS) was added, the microplates were incubated for 4 hours and cells lysed in 50% N,N-dimethylformamide containing 20% of SDS, pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular devices, CA, USA). The mean absorbance of medium controls was subtracted as a background. The viability of control cells was taken as 100% and the values of treated cells were calculated as a percentage of control. The $IC_{50}$ values were calculated from at least 3 independent experiments using linear regression of the dose-log response curves by SOFTmaxPro.

### Estimation of contents of cytochromes P450 1A1, 1B1 and 3A4 in neuroblastoma cells by Western blot

To determine the expression of CYP1A1, 1B1 and 3A4 proteins, cells were homogenized in RIPA buffer. Protein concentrations were assessed using the DC protein assay (Bio-Rad, Hercules, CA, USA) with serum albumin as a standard. 10–45 µg of extracted proteins were subjected to SDS-PAGE electrophoresis on a 10% gel. After migration, proteins were transferred to a nitrocellulose membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific anti-CYP1A1 (1:1000, Millipore, MA, USA) anti-CYP1B1 (1:500, AbCam, MA, USA) and anti-CYP3A4 (1:5000, AbD Serotec, Oxford, UK) rabbit polyclonal antibodies overnight at 4 °C. Membranes were washed and exposed to peroxidase-conjugated anti-IgG secondary antibody (1:3000, Bio-Rad, Hercules, CA, USA), and the antigen-antibody complex was visualized by enhanced chemiluminiscence's detection system according to the manufacturer's instructions (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA). Films (MEDIX XBU, Foma, Hradec Králové, Czech Republic) were scanned with a computerized image-analyzing system (ElfoMan 2.0, Ing. Semecký, Prague, Czech Republic).

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## Results

### Cytotoxicity of valproate and trichostatin A to human neuroblastoma cells

To evaluate the cytotoxicity of VPA and TSA to human neuroblastoma cells (UKF-NB-3, UKF-NB-4 and SK-N-AS), these cells were treated with increasing concentrations of both drugs (0–50 mM for VPA and 0–1 μM for TSA). We first determined the effect of VPA and TSA on growth of human neuroblastoma cell lines cultured for 72 hours in the presence of both drugs, using MTT assay. As shown in Figures 1 and 2, all three neuroblastoma cell lines were sensitive to VPA and TSA. Both drugs inhibited the growth of neuroblastoma cell lines in a dose-dependent manner. The $IC_{50}$ values for VPA and TSA calculated from the dose-log response curves are shown in Figures 1B and 2B.

Among the neuroblastoma cell lines tested in this study, the UKF-NB-3 cell line was the most sensitive to both drugs, with $IC_{50}$ values of 1.02 mM and 69.8 nM for VPA and TSA, respectively. The $IC_{50}$ values indicating the toxicity of VPA and TSA to UKF-NB-4 cells were similar to those found for the SK-N-AS cell line, being up to a 2.7-fold lower than for the UKF-NB-3 cell line (Figures 1 and 2). Nevertheless, the curves showing the viability of SK-N-AS cells under treatment with increasing concentrations of VPA and TSA significantly differed from those of UKF-NB-3 and UKF-NB-4 cell lines. At higher VPA and TSA concentrations, the sensitivity of SK-N-AS cells was much lower than that of other two neuroblastoma cell lines analyzed in this work.

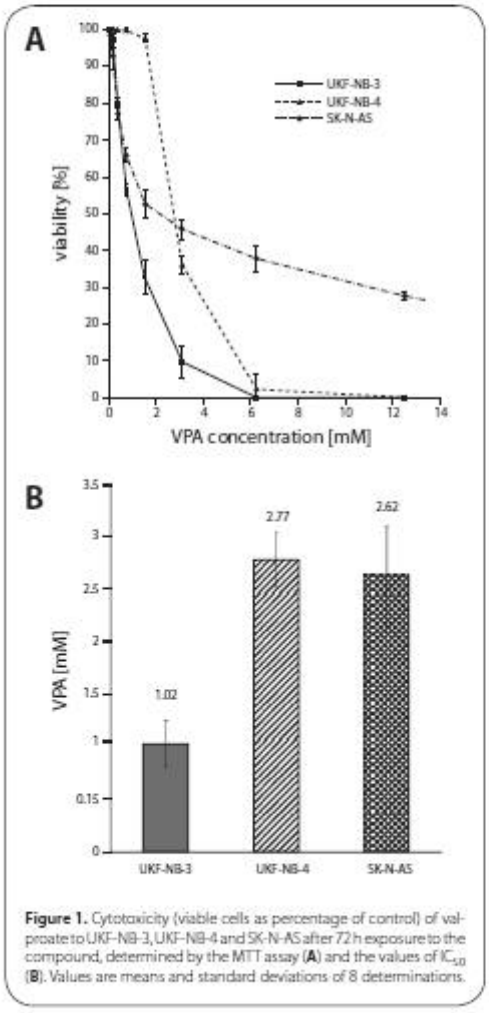


**Figure 1.** Cytotoxicity (viable cells as percentage of control) of valproate to UKF-NB-3, UKF-NB-4 and SK-N-AS after 72 h exposure to the compound, determined by the MTT assay (**A**) and the values of $IC_{50}$ (**B**). Values are means and standard deviations of 8 determinations.

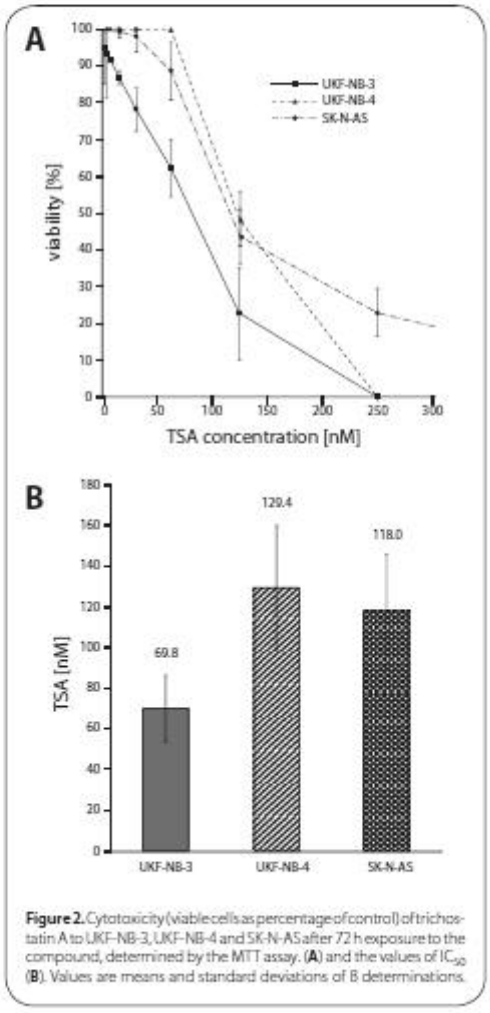


**Figure 2.** Cytotoxicity (viable cells as percentage of control) of trichostatin A to UKF-NB-3, UKF-NB-4 and SK-N-AS after 72 h exposure to the compound, determined by the MTT assay. (**A**) and the values of $IC_{50}$ (**B**). Values are means and standard deviations of 8 determinations.

**Effect of VPA and TSA on expression of cytochrome P450 1A1, 1B1 and 3A4 proteins**

Using Western blot analysis with antibodies raised against CYP1A1, 1B1 and 3A4, the effects of VPA and TSA on protein expression levels of these enzymes were analyzed in the tested neuroblastoma cell lines.

Expression of CYP1A1 protein in neuroblastoma UKF-NB-3 and UKF-NB-4 cells was elevated by increasing concentrations of VPA and/or TSA in a dose-dependent manner (Figure 3). A 1.7-, 4.0- and 8.1-fold increase in CYP1A1 expression was caused by treating the UKF-NB-3 cells for 48 hour with 0.5, 1.0 and 2.0 mM VPA, respectively, while lower, only up to a 1.7-fold increase in levels of this CYP was produced by VPA in UKF-NB-4 cells. In the SK-N-AS cells, even no effect of VPA on the CYP1A1 expression was detectable.

Similar effects on CYP1A1 expression in neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines were detected when cells were treated for 48 hours with TSA. Up to a 4.4-fold increase in expression levels of CYP1A1 was produced by 50–200 nM TSA in these cells (Figure 3). No effects of TSA on the expression of CYP1A1 protein in SK-N-AS were found.

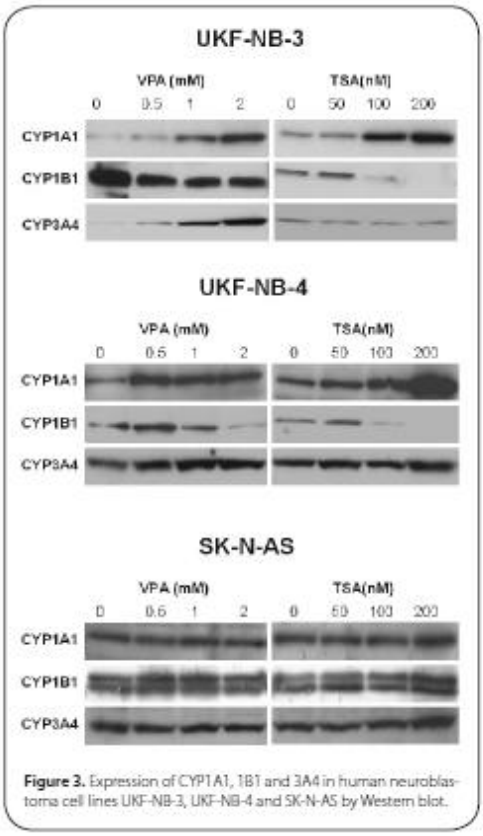


**Figure 3.** Expression of CYP1A1, 1B1 and 3A4 in human neuroblastoma cell lines UKF-NB-3, UKF-NB-4 and SK-N-AS by Western blot.

Expression of CYP1B1 protein was decreased in UKF-NB-3 and UKF-NB-4 cells after their 48-hour treatment with increasing concentrations of VPA and/or TSA, being decreased in a dose-dependent manner. Similar to CYP1A1, no effect of both HDAC inhibitors on expression of CYP1B1 was produced in SK-N-AS cells (Figure 3). Interestingly, two protein bands detectable by antibody against CYP1B1 were found in SK-N-AS cells.

In the case of the effects of VPA and TSA on expression of CYP3A4 protein in neuroblastoma cells, both these drugs essentially did not influence its expression in S-type UKF-NB-4 and SK-N-AS cell lines. The N-type of neuroblastoma cell line, UKF-NB-3, was only the exception; whereas increased concentrations of VPA increased CYP3A4 expression in this cell line, TSA had no effect (Figure 3).

## Discussion

The results of this work show that human neuroblastoma UKF-NB-3, UKF-NB-4 and SK-N-AS cell lines are sensitive to two tested HDAC inhibitors, VPA and TSA. In the case of VPA, its concentrations that were toxic to neuroblastoma cells are clinically applicable, since concentrations between 0.35–0.7 mM in patients serum are commonly therapeutically used (Duenas-Gonzalez *et al.*, 2007). The $IC_{50}$ values for VPA and TSA indicate that a UKF-NB-3 cell line was the most sensitive to both HDAC inhibitors, while their toxicity to the UKF-NB-4 and SK-N-AS cell lines was up to a 2.7-fold lower. Thus, the sensitivity to the two drugs seems to be related to the phenotype, with the S-type cells (UKF-NB-4 and SK-N-AS) being less sensitive than the N-type (UKF-NB-3), probably because of their partly lower capability of undergoing apoptosis (Servidei *et al.*, 2004). However, the results shown here indicate that it seems to be questionable to evaluate the toxic effects of chemicals to cells in culture using only the $IC_{50}$ values. The question arises, whether the $IC_{50}$ value is a real appropriate sensitivity marker. Namely, of the S-type neuroblastoma cells utilized in this study, the SK-N-AS cell line seems to be even less sensitive to VPA and TSA than the second S-type cell line, UKF-NB-4, even though the $IC_{50}$ values for VPA and TSA were similar for both these cell lines. At higher VPA and TSA concentrations, the sensitivity of SK-N-AS cells was much lower than that of UKF-NB-4. This less sensitive SK-N-AS line seems to be, at least in part, capable of overcoming treatment with VPA and TSA at concentrations that cause almost complete eradication of UKF-NB-4 cells. These results suggest that caution should be exerted to sort neuroblastoma cells into their types. Even in one type of neuroblastoma cells (S-type in this case), biological heterogeneity should be taken into account. This suggestion is also supported by further features found in this cell line. Namely, the SK-N-AS cell line behaves differently from the other S-type cell line, UKF-NB-4, from the point of view of the effects of VPA and TSA on CYP expression; no effects of both drugs was found on levels of individual CYP enzymes. Moreover, in this cell line, the two CYP1B1 protein bands

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were detectable by antibody against this CYP. Now, we can only speculate on the origin of the second protein band in SK-N-AS cells. The questions, whether it might follow from a degradation of the CYP1B1 protein in this cell line or it is the artifact caused by the method used (Western blot), remain to be answered.

The expression of all CYP enzymes analyzed in this work was modulated by VPA and TSA only in the N-type UKF-NB-3 cell line. Whereas the CYP1A1 enzyme was induced by both drugs, expression of CYP1B1 was depressed by both drugs. The CYP3A4 enzyme was increased by VPA, but TSA had no influence on the expression of this enzyme. The expression of CYP1A1 and 1B1 was also similarly affected by VPA and TSA in the UKF-NB-4 cell line, but no effect on expression levels of CYP3A4 was produced in this line. Similarity in response of UKF-NB-3 and UKF-NB-4 cells to the effects of VPA and TSA on CYP1A1 and 1B1 expression might probably be caused by their similar effects on state (degree) of acetylation of histones and, therefore, transcription activity. But differences between these two cell lines and particularly SK-N-AS cells in response to CYP enzyme expression and its affecting by VPA and TSA are still valuable. The question whether such differences are caused by the fact that cells vary in the broad spectrum of metabolic and signalling pathways that might also be affected by VPA and TSA in a different way, independently of a cell type (N- or S-type), remains to be answered. Further studies with these and other neuroblastoma cell lines and various HDAC inhibitors and broader spectrum of CYP enzymes have to be performed in order to shed more light on this field.

Since CYP enzymes are involved in biosynthesis and metabolism of many endogenous physiologically active substances and in biotransformation of xenobiotics with pharmacological and/or toxic effects (Myasodeova, 2008), a change in their expression might affect the cells significantly. In the case of oncology, the participation of CYPs in drug metabolism seems to be their most important role. A variety of CYP enzymes is involved in metabolism of a broad spectrum of drugs that can, moreover, either increase or decrease their expression levels. The finding that VPA and TSA are capable of inducing and depressing CYP enzyme expression in neuroblastoma cells (CYP1A1, 1B1 and 3A4 tested in our work) might have great importance. This feature might be utilized mainly in the combination therapy with other drugs whose pharmacological effects are dependent on their CYP-mediated metabolism. Such a study with one of these drugs, ellipticine, is under way in our laboratory.

## Acknowledgements

We thank Prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany) for providing cell lines. This work was supported by the Internal Grant Agency of Ministry of Health of the Czech Republic (grant NR9522-3/2007) and Ministry of Education of the Czech Republic (grant MSM0021620813 and RP MSMT 14/63).

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## Práce V: Expulsion of amplified MYCN from homogenously staining chromosomal regions in neuroblastoma cell lines after cultivation with cisplatin, doxorubicin, hydroxyurea, and vincristine

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CANCER GENETICS AND CYTOGENETICS

Short communication

# Expulsion of amplified MYCN from homogenously staining chromosomal regions in neuroblastoma cell lines after cultivation with cisplatin, doxorubicin, hydroxyurea, and vincristine

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**Abstract** Amplified *MYCN*, common in neuroblastomas, can be detected as double minutes (dmin) or homogenously staining chromosomal regions (hsr). Expulsion of amplified *MYCN* has only been described in dmin. We used hydroxyurea (HU), which accelerates the expulsion of amplified genes and cytostatics (used in neuroblastoma therapy), to describe *MYCN* amplification changes after chemotherapy. We used IMR-32, SK-N-AS, UKF-NB-2, UKF-NB-3, UKF-NB-4, and derived sublines resistant to doxorubicin, cisplatin, and vincristine. The loss of amplified *MYCN* copies was investigated using comparative genomic hybridization and by fluorescent in situ hybridization. We found expulsion of amplified *MYCN* from hsr in UKF-NB-4 and IMR-32 cell lines, and determined the exact number of amplified *MYCN* copies. After the first cultivation with HU, some amplified *MYCN* was lost. UKF-NB-4 lost 20 copies on average, and IMR-32 lost 15 copies ($P < 0.001$). After the second cultivation, cells without *MYCN* amplification were found. In comparison to sensitive cell lines, drug-resistant cell lines lost 17 copies on average. Our data show that expulsion of amplified *MYCN* genes is also possible from hsr and may be induced, not only by HU, but by other cytostatics as well. 

## 1. Introduction

Amplification of MYCN is a common event in neuroblastoma (NBL) and predicts a poor prognosis [1]. The human MYCN gene is located on the number 2 chromosome (2p24). Amplified MYCN can be detected by cytogenetics as extrachromosomal double minutes (dmin) or as homogenously staining regions (hsr), which are located on different chromosomes [2]. MYCN amplification in NBL usually yields 50–100 copies [2,3]. Elimination of amplified genes from tumor cells has been described in cell lines as well as in tumor samples collected from patients [4–9]. This phenomenon results through the formation of micronuclei which discharge the supernumerary gene copies to the cytoplasm [10]. Expulsion of amplified MYCN from NBL lines and tumor samples can be accelerated by hydroxyurea (HU) and has been described in dmin amplification, but not in hsr amplification [10–12]. Elimination of amplified MYC genes from human colon cancer cells has been shown to reduce tumorigenicity [11].

During treatment, a reduction of amplified MYCN in one NBL tumor sample was observed after chemotherapy, which consisted of a combination of doxorubicin (DOXO), cisplatin (CDDP), cyclophosphamide, and vincristine (VCR). The bone marrow–relapsed, drug-resistant clone showed decreased MYCN amplification [13]. Development of resistance is one of the major problems of cancer therapy. Resistance to one agent is often accompanied by resistance to other compounds and appears to be multifactorial [14]. Drug-resistant lines prepared by exposing parental cells to increasing concentrations of a particular drug are used as one of the in vitro models for the study of changes accompanying drug-resistance, regardless of genetic abnormalities, which are unrelated to drug-resistance because they are caused by multiple passages and/or by cytostatic drug treatment [14–16]. From this point, changes in MYCN amplification on drug-resistant cell lines were studied to substantiate and extend the findings of Kuroda et al. [13].

The aims of our study were to examine the amplification of the *MYCN* gene and its expulsion in (1) high-risk neuroblastoma (HR NBL) cell lines with hsr MYCN amplification and (2) resistant sublines, and (3) to observe differences in MYCN amplification in cell lines treated with either HU or CDDP.

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Table 1
Growth rate of tested cell lines during HU treatment

| Cell line | IMR-32 | SK-N-AS | UKF-NB-2 | UKF-NB-3 | UKF-NB-4 |
|---|---|---|---|---|---|
| NBL type | N | S | N | N | S |
| Type of amplification | HSR | - | hsr | hsr | hsr |
| HU concentration [μmol/L] | 100 | 100 | 100 | 100 | 100 |
| 1st week | ++ | +++ | +++ | ++ | +++ |
| 2nd week | ++ | +++ | + | + | +++ |
| 3rd week | + | +++ | + | + | +++ |
| 4th week | + | +++ | + | ++ | ++ |
| 5th week | + | +++ | + | +++ | ++ |
| 6th week | - | +++ | - | ++ | ++ |
| 7th week | - | +++ | - | ++ | ++ |
| 8th week | - | +++ | - | +++ | + |
| 9th week | - | +++ | - | ++ | + |
| 10th week | - | +++ | - | ++ | + |
| 11th week | - | +++ | - | ++ | +++ |

+++, growth (two passages per week); ++, slow growth (one passage per week); +, living cells (only change of fresh medium containing HU without passage); −, cell death.

## 2. Materials and methods

### 2.1. Cell lines

Cell lines UKF-NB-2, UKF-NB-3, and UKF-NB-4 were established from bone marrow metastases of three HR NBL with MYCN amplification, and UKF-NB-4 was derived from material harvested after recurrence. The IMR-32 cell line was established from an abdominal mass. The VCR-, DOXO-, and CDDP-resistant sublines, designated UKF-NB-4$^{CDDP}$, UKF-NB-4$^{DOXO}$, UKF-NB-4$^{VCR}$ and IMR-32$^{DOXO}$, were prepared by incubation of parental cells with increasing concentrations of the respective drugs [17]. The lines were kindly provided by Dr. J. Cinatl, Jr. (Institute for Medical Virology, Johann Wolfgang Goethe University Hospital, Frankfurt am Mein, Germany). Cells were grown in Iscove's modified Dulbecco's medium with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). The medium for resistant sublines contained the appropriate drug (1,000 ng/mL CDDP, 50 ng/mL VCR, and

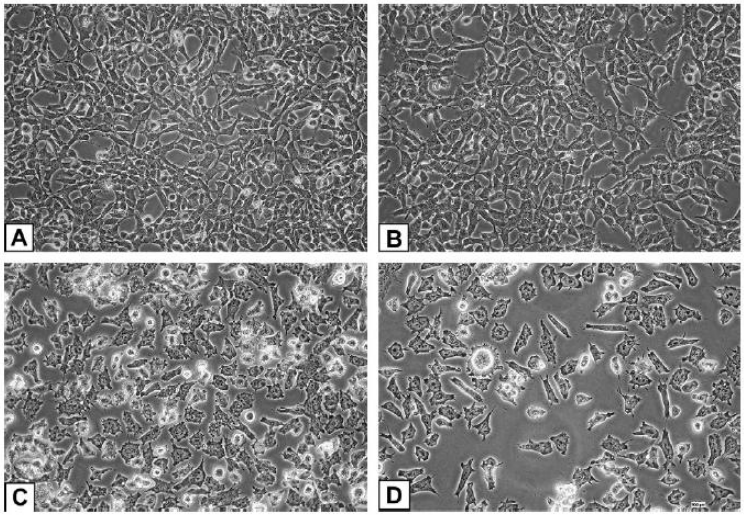


Fig. 1. Morphology of growing cell lines during cultivation with HU. The UKF-NB-3 cell line represents the N-type of NBL (A). Cells displayed no change in morphology (B) during cultivation with HU compared to growing UKF-NB-3 cultivated without HU. UKF-NB-4 represents the S-type of NBL (C). Cells cultivated with HU showed minor changes in morphology. In the UKF-NB-4 cultivated with HU, it was often possible to find cells with an altered (flattened and extended) shape (D). Magnification: ×20.

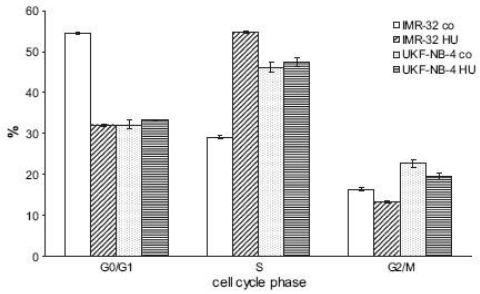


Fig. 2. Effect of HU on cell cycle distribution. Cells were treated with vehicle (CO) (UKF-NB-4$^{CO}$ and IMR-32$^{CO}$) or HU for 3 days (UKF-NB-4$^{HU}$ and IMR-32$^{HU}$), and cell cycle distribution was assessed using flow cytometry. $N = 3$, values are means ± S.D.

50 ng/mL DOXO). The SK-N-AS cell line, which does not amplify MYCN, was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

### 2.2. Drugs

Solutions of HU (Sigma-Aldrich, Deisenhofen, Germany), VCR (Gedeon Richter Ltd., Budapest, Hungary), DOXO, and CDDP (EBEWE Pharma Ges.m.b.H. Nfg. KG, Unterach, Austria) were prepared and stored according to the manufacturer's instructions.

### 2.3. Hydroxyurea treatment

All tested lines were exposed to 100-µmol/L concentrations of HU, as described by the group in Ambros et al. [12]. Cells were controlled microscopically every 2 days and subcultured into a new vessel (passage) if necessary (usually once a week). If cells grew slowly with HU, the HU medium was changed without passage at weekly intervals.

### 2.4. Viability and cell cycle analysis

Viability was evaluated by trypan blue exclusion and measured using an FACSCalibur cytometer (Becton-Dickinson, San Jose, CA) [18]. For cell cycle analysis, cells were stained with propidium iodide using the DNA-Prep Reagent System (Beckman Coulter, Fullerton, CA), measured with a cytometer, and analyzed using ModFit LT software (Verity Software House, Topsham, ME).

### 2.5. Isolation of DNA and comparative genomic hybridization (CGH)

Genomic DNA was extracted from cells using a Wizard DNA Isolation Kit (Promega, Madison, WI) according to the manufacturer's instructions. Concentrations were measured with a Biomate 3 spectrophotometer (Thermo Scientific, Waltham, MA). CGH was performed as described previously [14].

### 2.6. Fluorescent in situ hybridization (FISH)

Cells were incubated in hypotonic solution and fixed with methanol and acetic acid. Cell suspensions were applied to glass specimen slides and heated. After cooling, the specimen slides were incubated with RNase (Promega, Madison, WI), washed twice with standard saline citrate (SSC) wash buffer (Oncor, Gaithersburg, MD), dehydrated with series of increasing ethanol concentrations, and dried. Then the slides were incubated in a pepsin solution (Sigma, Steinheim, Germany), washed, fixed in formaldehyde, washed again, dehydrated, dried, denatured in 70% formamide/2× SSC (Roche, Mannheim, Germany) at 72 °C, dehydrated, and dried. On MYCN (2p24)/LAF (2q11), FISH probes (Kreatech Biotechnology B.V., Amsterdam, The Netherlands) were denatured at 74 °C and applied to the slides, covered with coverslips, and incubated overnight at 37 °C. On the day 2, the coverslips were removed and the slides were washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI)/Antifade (Kreatech Biotechnology B.V.), and recovered with a glass coverslip. Using a fluorescence microscope (Olympus AX70), copies of the *MYCN* gene and 2q11 were counted in the nuclei of 200 cells. The amount of no. 2 chromosomes (i.e., 2q11 signals) was subtracted from the number of *MYCN* copies for each nucleus observed.

### 2.7. Statistical analysis

All numerical data are presented as the mean ± standard deviations, and were analyzed statistically using the Student's *t* test and chi-square test with a 2 × 2 contingency.

## 3. Results

### 3.1. Impact of HU treatment on the growth and viability of different NBL cell lines

Five lines were exposed to 100-µmol/L concentrations of HU for up to 11 weeks (5–11 weeks). We observed marked variation in the reaction pattern of the different lines after exposure to HU (Table 1). All MYCN-amplified lines had decreased growth rates during cultivation, and the IMR-32, UKF-NB-2 and UKF-NB-3 lines stopped growing after 1 or 2 weeks. The IMR-32 and UKF-NB-2 lines died out slowly. The UKF-NB-3 line didn't grow for 2 weeks and then became resistant (data not shown) and had stable growth, although slower than controls. (Fig. 1, A and B). Only the UKF-NB-4 line grew for the first 3 weeks without any change. We found increased numbers of cells in the G0/G1 phase in UKF-NB-4, while we found S phase arrest in IMR-32 after cell growth had stopped (Fig. 2). After 3 weeks, the UKF-NB-4 growth rate slowed, and in the 8th week, growth stopped altogether. After the 10th week, cells resumed growth, and by the 11th week, cells were growing normally again. UKF-NB-4 cultivated with HU showed few morphologic changes compared to controls (Fig. 1, C and D). The SK-N-AS line showed no change in growth rate

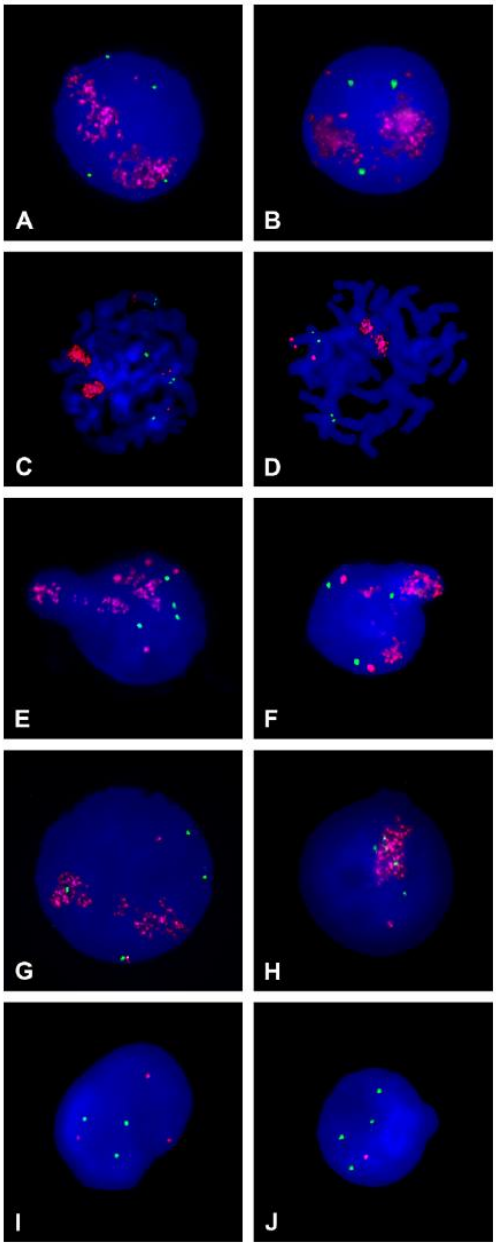


Fig. 3. Expulsion of amplified MYCN from hsr. FISH displays nuclei from the UKF-NB-4 cell line (A) with two hsrs and the IMR-32 cell line (B) with two hsr. Chromosomes of the UKF-NB-4 cell line with two hsr translocated outside the number 2 chromosome (C), and chromosomes of UKF-NB-4 after 11 passages with HU, which experiences smaller hsr than the sensitive UKF-NB-4 line (D). Expulsion of amplified MYCN from the nuclei in UKF-NB-4 line could be found after cultivation with HU (11th passage; E). Cells that expelled MYCN could also be found in the nuclei of the IMR-32 line after the first passage with HU (F). Many cells have two hsr (G) or only one hsr (H) in their nucleus after cultivation with HU. hsr are disrupted by the activity of HU and have fewer MYCN copies (G and H). All hsr were expelled after cultivation with HU (I and J). Nuclei without any hsr were very rare in the IMR-32 cell line cultivated with HU because IMR-32 cells were not capable of prolonged growth in the presence of HU. Magnification: ×100.

or morphology during cultivation with HU. Viability of UKF-NB-4 (89%) and IMR-32 (95%) was decreased by the third day of cultivation with HU compared to untreated controls ($t$ test, UKF-NB-4, $P < 0.001$; IMR-32, $P < 0.05$).

### 3.2. Changes in the degree of the MYCN amplification in UKF-NB-4 and IMR-32 cell lines after HU treatment

The number of MYCN copies in lines with MYCN amplification were verified using FISH; this allowed determination of the exact number of MYCN copies in each nucleus, unlike with other molecular techniques (Fig. 3) [19]. After the first HU passage, there was a decrease in the number of MYCN copies in the UKF-NB-4 and IMR-32 lines ($t$ test, $P < 0.001$ both lines; Fig. 4). The IMR-32 line died out during the third passage, which is why only the first and second passages were counted. The UKF-NB-4 cell line grew in the presence of HU for a longer period of time. We counted MYCN in the 6th passage (3rd week) and the 10th passage (7th week). During cultivation with HU, the number of MYCN copies varied, but it was always lower than the number in the control without HU. In both counted passages, we found cells with only four MYCN copies; i.e., without any excessive copies. In the 6th passage, two cells without any excessive MYCN copy were found, and in the 10th passage, only 1 such cell was found out of 200 cells (Fig. 5). During cultivation with HU, cells lost not only separate MYCN copies, but also the entire hsr (Table 2). MYCN expulsion from cells cultivated with HU was also observed (Fig. 3; Table 2). After the 10th passage, cells numbers stopped increasing, but the existing cells remained alive. During the depressed growth phase, cells started to restore the lost amplified MYCN copies (the average number of MYCN copies in the 10th passage was 34, and the average number in the 11th was 46; $t$ test, $P < 0.001$; Fig. 4). We found signs of MYCN expulsions more frequently in the second HU passage in IMR-32 (but not in the first HU passage) than in the IMR-32 cultivated in medium without HU. Line IMR-32$^{DOXO}$ did not have more cells with MYCN expulsion than the parent line. UKF-NB-4 cultivated with HU had more cells with signs of expulsion in the 1st, 10th, and 11th, but not in the 6th passage (Table 2).

### 3.3. Changes in the degree of the MYCN amplification in resistant sublines

The average number of MYCN gene copies in resistant sublines derived from UKF-NB-4 and IMR-32 were decreased

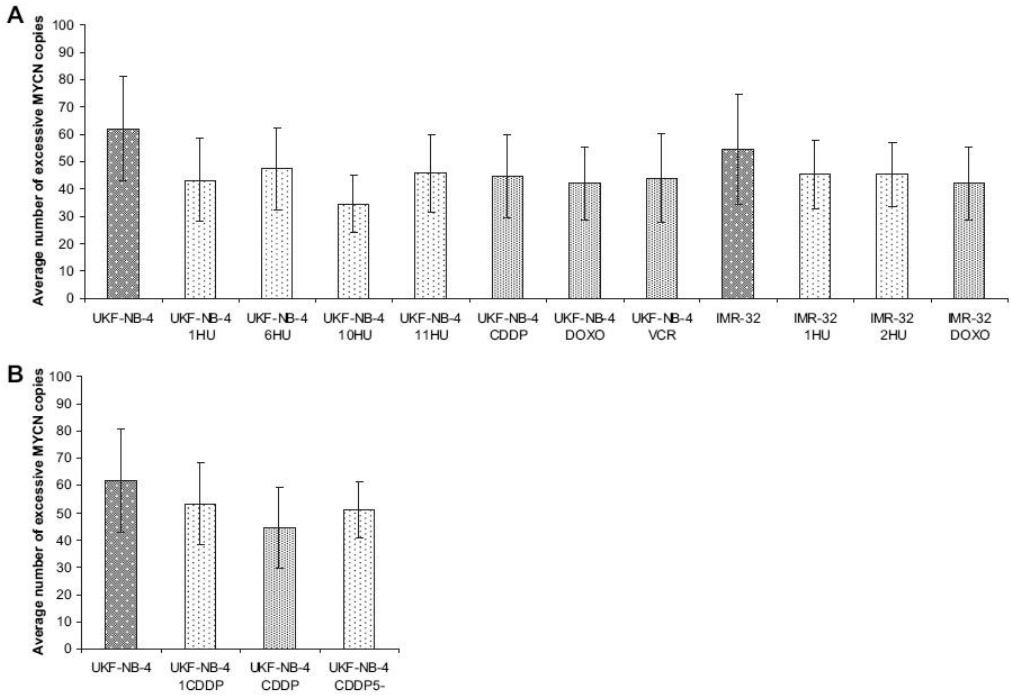


Fig. 4. Average number of excessive *MYCN* gene copies in tested cell lines. Copies of the *MYCN* gene were counted in 200 nuclei of each cell line studied. The number of number 2 chromosomes, established using a centromere probe, was subtracted from the number of MYCN copies to get the number of extra MYCN copies. The arithmetic means of the excess copies of MYCN as well as the standard deviations were calculated for each cell line (*t* test, $p < 0.001$).
UKF-NB-4$^{1(6,\ 10,\ 11)HU}$ – cell line UKF-NB-4 in the 1st (6th, 10th, 11th) passage with HU
UKF-NB-4$^{1CDDP}$ – cell line UKF-NB-4 in the first passage with CDDP
UKF-NB-4$^{CDDP\ (DOXO,\ VCR)}$ – cell line UKF-NB-4 resistant to cisplatin (doxorubicin, vincristine)
UKF-NB-4$^{CDDP5-}$ – cell line UKF-NB-4 CDDP cultivated five passages without CDDP
IMR-32$^{1(2)HU}$ – cell line IMR-32 in the first (second) passage with HU
IMR-32$^{DOXO}$ – cell line IMR-32 resistant to doxorubicin

compared to parental lines (*t* test, $P < 0.001$; Fig. 4). The distribution of cells, based on the number of excessive copies, is shown in Figs. 5 and 6. In the sensitive cell lines (UKF-NB-4 and IMR-32), a Gaussian-like distribution was observed, and the same distribution of the MYCN copy number could also be seen in resistant cells, but it was shifted to the left by about 20 copies in UKF-NB-4–resistant lines and by about 15 copies in IMR-32$^{DOXO}$. Out of 200 nuclei, the UKF-NB-4 line had 4 cells and the IMR-32 line had 8 cells with amplification greater than 100 copies per nucleus. These cells were only sporadically present (0–2 cells) in UKF-NB-4– and IMR-32–resistant sublines.

### *3.4. Influence of CDDP on MYCN amplification*

We compared the effect of HU and CDDP on MYCN status. A one-shot addition of 1,000 ng/mL of CDDP decreased the average number of MYCN copies from 62 to 53 ($P < 0.005$; Fig. 4). During prolonged cultivation with CDDP, the average number of extra MYCN copies in UKF-NB-4$^{CDDP}$ was 45, and the MYCN status remained stable (data not shown). After 5 weeks of cultivation without CDDP, the average number increased from 45 to 51 copies in UKF-NB-4$^{CDDP}$ ($P < 0.01$). MYCN expulsions were visible after incubation with CDDP, but were not as common as with HU. By the third day of cultivation with CDDP, viability of UKF-NB-4 cells had decreased (controls and cells with CDDP, *t* test, $P < 0.001$).

## 4. Discussion

The mechanism of MYCN amplification in NBL is still unknown, but it seems to agree well with the

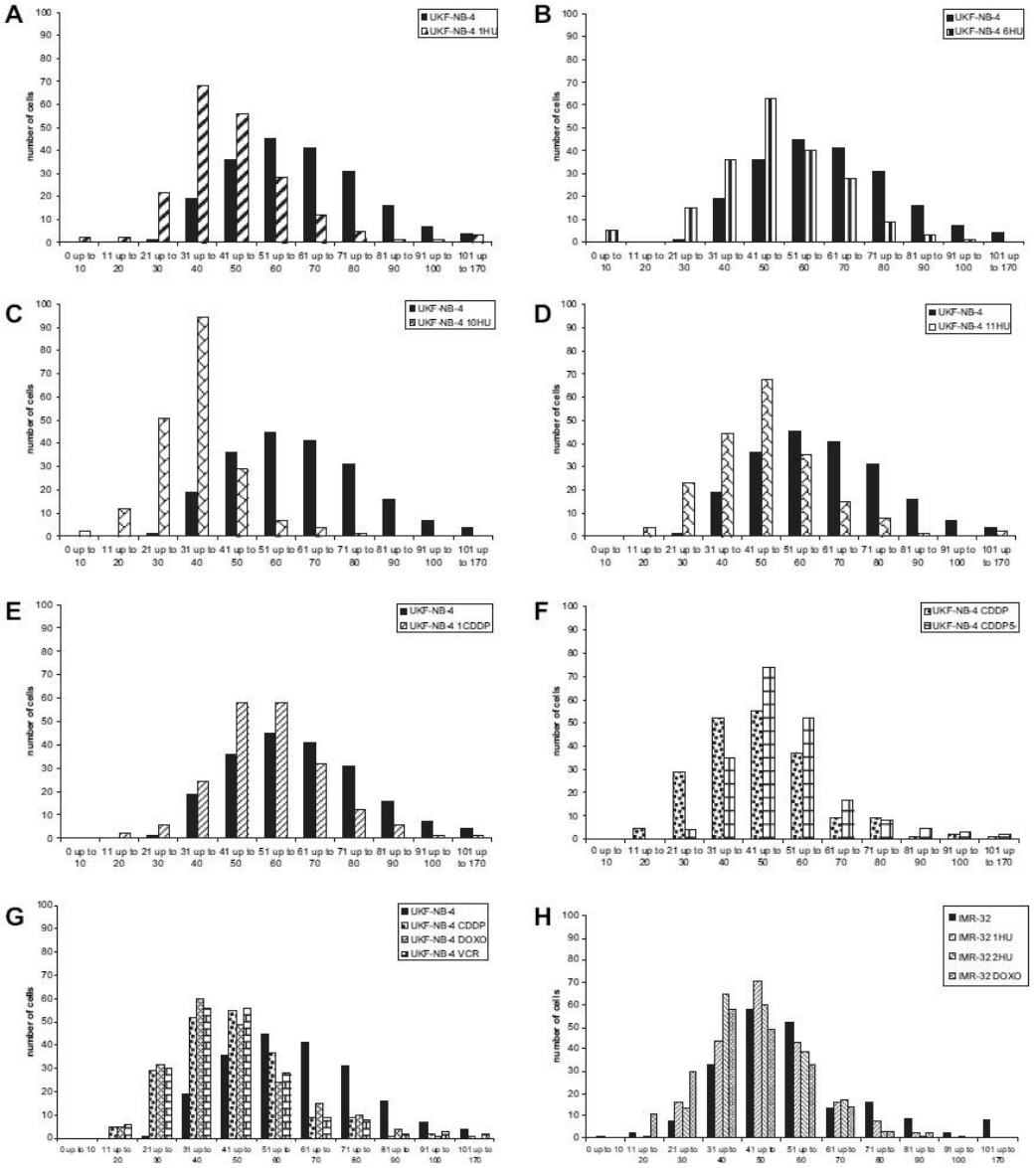


Fig. 5. Distribution of cells according to the number of excessive *MYCN* copies. In the parental-sensitive cell lines (UKF-NB-4 and IMR-32), a Gaussian-like distribution having few cells with high amplification (more than100 copies per cell) could be seen. These cells are either not present or are present sporadically in drug-resistant cell lines or cell lines cultivated with cytostatics. The Gaussian-like distribution can also be seen in cell lines cultivated with cytostatics or in drug-resistant sublines, but the distribution is shifted to the left by about 20 copies.

UKF-NB-4$^{1(6,\ 10,\ 11)HU}$ – cell line UKF-NB-4 in the 1st (6th, 10th, 11th) passage with HU

UKF-NB-4$^{1CDDP}$ – cell line UKF-NB-4 in the first passage with CDDP

UKF-NB-4$^{CDDP\ (DOXO,\ VCR)}$ – cell line UKF-NB-4 resistant to cisplatin (doxorubicin, vincristine)

UKF-NB-4$^{CDDP5-}$ – cell line UKF-NB-4 CDDP cultivated five passages without CDDP

IMR-32$^{1(2)HU}$ – cell line IMR-32 in the first (second) passage with HU

IMR-32$^{DOXO}$ – cell line IMR-32 resistant to doxorubicin

Table 2
Number of cells expelling MYCN and number of hsrs in tested cell lines

| Cell line | Observed expulsions | 2 hsr | 1 hsr | Ratio |
|---|---|---|---|---|
| IMR-32 | 53/200 | 158 | 42 | 3.76:1 |
| IMR-32$^{1HU}$ | 67/200NS | 156 | 44 | 3.54:1 NS |
| IMR-32$^{2HU}$ | 94/200*** | 155 | 45 | 3.44:1 NS |
| IMR-32$^{DOXO}$ | 64/200NS | 139 | 61 | 2.28:1 * |
| UKF-NB-4 | 54/200 | 150 | 50 | 3:1 |
| UKF-NB-4$^{1HU}$ | 92/200*** | 144 | 56 | 2.57:1 NS |
| UKF-NB-4$^{6HU}$ | 51/200NS | 135 | 65 | 2.08:1 NS |
| UKF-NB-4$^{10HU}$ | 89/200*** | 128 | 72 | 1.78:1 * |
| UKF-NB-4$^{11HU}$ | 119/200*** | 137 | 63 | 2.17:1 NS |

*Abbreviations*: 2 hsr, no. of cells with two hsr; 1 hsr, number of cells with one hsr; NS, not significant; *$P < 0.05$; *** $P < 0.005$; ratio, 2 hsr/1 hsr.

Compared with untreated UKF-NB-4 or IMR-32, chi-square test using a $2 \times 2$ contingency table.

replication–excision model. The DNA undergoes another round of replication only locally, and the extrareplicated DNA are excised and persist as dmin. This extrachromosomal DNA can integrate into any chromosome and may undergo several cycles of amplification in situ [2]. Extrachromosomally amplified MYCN, known as dmin, can be expelled through micronuclei formation. This expulsion is increased by HU [10–12], but there is no information so far regarding whether intrachromosomally amplified MYCN can be expelled. Von Hoff and associates [11] reported a decrease in the number of dmin MYC copies, as determined by Southern blotting in HL-60 cell sublines after treatment with HU; no change was reported for hsr MYC copies. Villa et al. found dmin MYC extrusion from AML (acute myeloid leukemia) blasts in three patients [20]. The group from Ambros et al. found that inducible loss of amplified MYCN was restricted to lines with dmin and was not found in cells with hsr [10,12]. In our study, however, we found expelled amplified MYCN copies from hsr in the UKF-NB-4 and IMR-32 lines. UKF-NB-4 was able to grow in the presence of HU with high viability and expelled MYCN, while other MYCN amplified cell lines except UKF-NB-3 grew for only 1 or 2 weeks and then died.

It is known that MYCN copies are possible to count in dmin and hsr using FISH [19]; although it is not possible to count the MYCN copy number in all cell lines with hsr because some lines with hsr amplification have MYCN copies in one or two groupings, making it impossible to count the number of copies accurately. For example, it was not possible to count MYCN copies in UKF-NB-3, but MYCN copies were countable in the UKF-NB-3$^{CDDP}$ line. The number of MYCN copies in IMR-32 (55 copies), which were counted using FISH, is in agreement with the number of MYCN copies measured (53 copies), using real-time PCR, by De Preter et al. [21]. MYCN copies were numbered in the 1st, 6th, 10th, and 11th passages of the UKF-NB-4, as well as in the 1st and 2nd passages of IMR-32. Our results show that the most significant decrease occurred during the first run of cultivation with HU or CDDP (Figs. 4, A and B, and 5, A and E).

All resistant sublines had hsr amplification, and the average number of MYCN copies compared with the parental line decreased [22]. Expelled MYCN copies could be seen around the nucleus. Using CGH, we found a relative loss of 2p24, which verified the decreased number of amplified MYCN gene copies in the resistant lines derived from UKF-NB-4 [14] and IMR-32. In the UKF-NB-4$^{CDDP}$, a relative loss of 2p24, which was confirmed by CGH, corresponds with Yasuno's findings [23]. We assume that cells with a high degree of MYCN amplification may have been cytostatically killed, or these cells could expel large numbers of MYCN copies from nuclei, because we did not find any nuclei with more than 100 extra MYCN copies in resistant cells (Fig. 5). Increased numbers of cells with signs of MYCN expulsion in cells cultivated with HU compared to controls without HU proved that MYCN expulsion can cause a decrease in MYCN copies in cells cultivated with HU. The number of copies counted in the 11th passage with HU corresponds to the number of copies in resistant sublines derived from UKF-NB-4. UKF-NB-4 in the 11th passage with HU had an average of 46 extra copies and other resistant UKF-NB-4 sublines had an average of 43 (UKF-NB-4$^{DOXO}$), 44 (UKF-NB-4$^{VCR}$), and 45 (UKF-NB-4$^{CDDP}$) copies. We surmised that resistant cells reduced the number of excessive MYCN copies to a value of about 45 extra copies when cultivated in a cytostatic medium.

The decrease in MYCN amplification in resistant lines or during cultivation with CDDP seems to be important. Decreased MYCN amplification in NBL lines established from the same patient before and after chemotherapy have been described [13]. Mizushima reported a nonsignificant decrease in MYCN amplification in two small-cell lung carcinoma lines resistant to CDDP [24]. We used FISH analysis, which measured the precise number of gene copies and is more accurate than the $^{32}P$ labeling used by Mizushima's group [24]. Our findings agree with the results of Yasuno et al., who described a decrease in the number of MYCN amplified gene copies measured by Southern blotting in NBL CDDP-resistant lines; the sensitive line had 50 copies and the CDDP-resistant line had only 6 copies [23].

Three distinct cell types of NBL cell lines termed N (neuroblastic), S (Schwann-like or stromal type), and I (intermediate type) have been described. The biologic differences between S and N subtypes may be of clinical relevance [25]. S-type cells may be better able to persist after chemotherapy [26]. From the four MYCN-amplified cell lines used, the S-type cell line (UKF-NB-4) had a greater ability to grow in the presence of HU compared to the three N-type lines (IMR-32, UKF-NB-2, and UKF-NB-3). These results support the argument that S-type cells can potentially give rise to resistant clones in NBL patients [26]. The reduced sensitivity of UKF-NB-4 (established after recurrence) to HU could also been induced by previous chemotherapy.

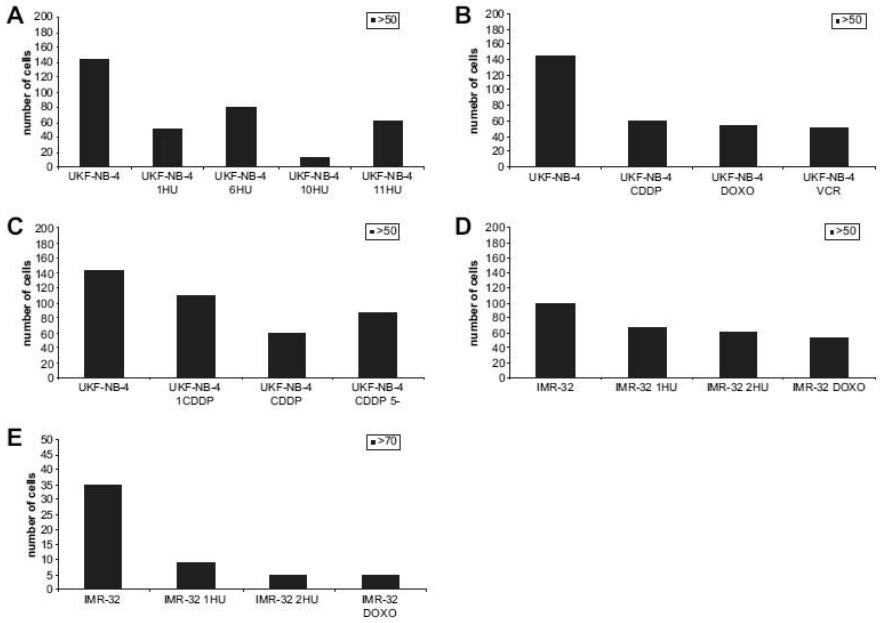


Fig. 6. Distribution of cells according to the excessive MYCN copies. In the UKF-NB-4 cell line, the counted nuclei were separated into two groups of either 50 copies or more than 50 copies. Differences in the number of MYCN copies between sensitive cell lines and cell lines cultivated with cytostatics are highly significant ($\chi^2$, $P < 0.001$). In the comparison between cell line UKF-NB-4$^{CDDP5-}$ and UKF-NB-4$^{CDDP}$, the results, while still statistically significant, are less dramatic ($\chi^2$, $P < 0.01$). In the IMR-32 cell line, the number of extra MYCN copies counted in 200 cell nuclei was also separated into groups of 50 copies or more than 50 copies. Changes in the group of cells with more than 50 copies was statistically highly significant in the second passage with HU and in the cell line resistant to DOXO ($\chi^2$, $P < 0.001$; D), while the first passage with HU was statistically significant, but less so ($\chi^2$, $P < 0.01$). If we separated nuclei into groups of 70 copies or more than 70 copies (E), it was possible to get a statistically highly significant change even in the first passage with HU ($\chi^2$, $P < 0.001$).
UKF-NB-4$^{1(6,\ 10,\ 11)HU}$ – cell line UKF-NB-4 in the 1st (6th, 10th, 11th) passage with HU
UKF-NB-4$^{1CDDP}$ – cell line UKF-NB-4 in the first passage with CDDP
UKF-NB-4$^{CDDP\ (DOXO,\ VCR)}$ – cell line UKF-NB-4 resistant to cisplatin (doxorubicin, vincristine)
UKF-NB-4$^{CDDP5-}$ – cell line UKF-NB-4 CDDP cultivated five passages without CDDP
IMR-32$^{1(2)HU}$ – cell line IMR-32 in the first (second) passage with HU
IMR-32$^{DOXO}$ – cell line IMR-32 resistant to doxorubicin

Our results with cell line SK-N-AS support the conclusions of Ambros et al., that lines without MYCN amplification are resistant to HU [12]. Therefore we can speculate that the sensitivity of NBL cells may also depend on the MYCN status.

In summary, IMR-32 and UKF-NB-4 showed a decrease in the number of MYCN copies and expulsion of amplified MYCN from nuclei. NBL cells could expel both single MYCN copies or whole hsrs from nuclei during cultivation with HU or CDDP in certain cell lines, and it is reasonable to speculate that a similar phenomenon occurs in HR NBL patients. To the best of our knowledge, no information regarding the expulsion of amplified MYCN genes from hsr was found in the literature. Resistant sublines have fewer MYCN amplification copies and fewer hsrs than sensitive ones.

**Acknowledgments**

We thank J. Cinatl Jr. (Institute for Medical Virology, Johann Wolfgang Goethe University Hospital, Frankfurt am Mein, Germany), who kindly provided cell lines. This research was supported by the Grant Agency of Charles University (grant 7926/2007) and by the Ministry of Education, Youth and Sports of the Czech Republic (grant MSM0021620813).

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## Práce VI: Cisplatin impacts the number of MYCN gene copies and corresponding expression in human neuroblastoma cell lines

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Article Type: Research Paper

Keywords: high risk neuroblastoma cell line; drug-resistance; MYCN amplification; 2p gain; MYCN expression; cisplatin.

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Abstract: Neuroblastoma is a tumor accounting for approximately 10% of all childhood malignancies and 50% of all childhood cancer deaths and is derived from cells originating from the neural crest. MYCN gene copy number variation represents the most important prognostic factor in neuroblastoma. Prognostic significance of MYCN gene expression is more complicated and depends on other factors such as MYCN gene copy number status.
Here we addressed MYCN gene copy numbers (gain and amplification) and corresponding mRNA expression in relation to the cisplatin treatment in three human neuroblastoma cell lines (UKF-NB-3, UKF-NB-4 and SK-N-AS) and their cisplatin-resistant counterparts. We examined MYCN gene status using fluorescent in situ hybridization and corresponding mRNA expression using real-time RT PCR. In MYCN-amplified neuroblastoma cells (UKF-NB-3 and UKF-NB-4) we observed a decreased number of MYCN copies during cisplatin treatment, whereas MYCN-non amplified SK-N-AS cells revealed an increased number of MYCN gene copies caused by a 2p gain. Sensitive neuroblastoma cells exhibited decreased MYCN expression following the cultivation with cisplatin. On the contrary, cisplatin resistant cells increased MYCN expression irrespective of the number of MYCN copies or concentration of cisplatin in the medium.
We postulated that MYCN overexpression may participate in cisplatin resistance caused by long-term cultivation with cisplatin. Our findings may contribute to the understanding of drug-resistance in connection with MYCN amplification and expression in neuroblastoma cells and may be of importance since targeting of MYCN is being tested as neuroblastoma therapy.

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Cover Letter

L.H.F. Mullenders
*Editor*
Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis

Dear Editor,

We would like to ask you for consideration of our manuscript, "Cisplatin impacts the number of *MYCN* gene copies and corresponding expression in human neuroblastoma cell lines" for Medical Oncology. The manuscript has not been previously published nor is being considered for publication elsewhere. It has been approved by all authors and by the responsible authorities of Department of Pediatric Hematology and Oncology, 2nd Medical Faculty and University Hospital Motol, Prague and by the responsible authorities at the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague.

We believe that changes in *MYCN* gene are still of importance in relation to chemotherapy of neuroblastoma. Therefore we addressed in our study *MYCN* gene copy numbers and corresponding mRNA expression in relation to cisplatin treatment in high risk neuroblastoma cell lines and from them the derived cisplatin-resistant counterparts. We found that resistant neuroblastoma cells with *MYCN* amplification decreased the number of *MYCN* copies and, on the contrary, increased the MYCN expression whereas the *MYCN*-non amplified cells revealed after cisplatin treatment an increased number of *MYCN* gene copies caused by a 2p gain and also increased MYCN expression. Our finding of the 2p gain in neuroblastoma in connection with drug-resistance is reported for the first time.

Thank you very much for your kind consideration.

Yours Sincerely,

_____
Dr. Pavel Prochazka
on behalf of all co-authors

*Highlights

## Highlights

► Cisplatin cause changes in the number of *MYCN* copies in neuroblastoma cells

► Long-term cultivation with cisplatin increases MYCN expression in neuroblastoma cells

► Short-term cultivation with cisplatin decreases MYCN expression in cisplatin-sensitive neuroblastoma cells

► High MYCN expression in cisplatin-resistant neuroblastoma cells does not depend on presence of CDDP

*Conflict of Interest Statement

## Conflict of interest statement

The authors have no conflicts of interest.

# Cisplatin impacts the number of *MYCN* gene copies and corresponding expression in human neuroblastoma cell lines

Pavel Prochazka [a,b,*], Jan Hrabeta [a], Simon Cipro [a], Ales Vicha [a], Eva Stejskalova [a], Pavel Vodicka [b,c], Tomas Eckschlager [a]

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## Abstract

Neuroblastoma is a tumor accounting for approximately 10% of all childhood malignancies and 50% of all childhood cancer deaths and is derived from cells originating from the neural crest. *MYCN* gene copy number variation represents the most important prognostic factor in neuroblastoma. Prognostic significance of *MYCN* gene expression is more complicated and depends on other factors such as *MYCN* gene copy number status.

Here we addressed *MYCN* gene copy numbers (gain and amplification) and corresponding mRNA expression in relation to the cisplatin treatment in three human neuroblastoma cell lines (UKF-NB-3, UKF-NB-4 and SK-N-AS) and their cisplatin-resistant counterparts. We examined *MYCN* gene status using fluorescent in situ hybridization and corresponding mRNA expression using real-time RT PCR. In *MYCN*-amplified neuroblastoma cells (UKF-NB-3 and UKF-NB-4) we observed a decreased number of *MYCN* copies during cisplatin treatment, whereas *MYCN*-non amplified SK-N-AS cells revealed an increased number of *MYCN* gene copies caused by a 2p gain. Sensitive neuroblastoma cells exhibited decreased MYCN expression following the cultivation with cisplatin. On the contrary, cisplatin resistant cells increased MYCN expression irrespective of the number of *MYCN* copies or concentration of cisplatin in the medium.

We postulated that MYCN overexpression may participate in cisplatin resistance caused by long-term cultivation with cisplatin. Our findings may contribute to the understanding of drug-resistance in connection with *MYCN* amplification and expression in neuroblastoma cells and may be of importance since targeting of *MYCN* is being tested as neuroblastoma therapy.

## *Key Words:*

high risk neuroblastoma cell line, drug-resistance, MYCN amplification, 2p gain, MYCN expression, cisplatin

## 1. Introduction

Neuroblastoma (NBL), a tumor of the sympathetic nervous system, is the most common solid extracranial childhood tumor. *MYCN* (V-myc myelocytomatosis viral related oncogene, neuroblastoma derived) was the first amplified oncogene that turned out to be of high clinical significance due to its association with the most aggressive form of NBL [1,2]. *MYCN* belongs to the family of *MYC* genes (*MYC*, *MYCN*, *MYCL*) which encode transcription regulators that participate in the control of both cell growth and apoptosis depending on the cellular context [3]. In healthy humans, MYCN is expressed only in some embryonal tissues and in adult cells there is its expression only very low or missing [4]. The human *MYCN* gene is normally located on the short arm of the chromosome 2 (2p24). The term 2p24 gain is suggested as a descriptive generic term for *MYCN* signal numbers exceeding up to a 4-fold the number of reference signals on the chromosomal arm 2q. This pattern could reflect either a gain of short arms of chromosome 2 (2p gain) or a gain of the *MYCN* gene (*MYCN* gain) which is not an independent prognostic factor in NBL [5] and could indicate an "incipient" *MYCN* amplification [6]. *MYCN* amplification is defined as more than 4-fold increase in the *MYCN* signal number compared to the reference probe, located on the chromosome 2q [6]. Amplified *MYCN* can take the form of either extrachromosomal double minutes or as homogenously staining chromosomal regions (HSRs), which are usually located on chromosomes other than 2p (normal *MYCN* location) [7].

Three morphologically distinct cell types termed N (neuroblastic type), S (Schwann-like or stromal type) and I (intermediate type) occur in NBL. The biological differences between S and N subtypes may be of clinical relevance [8]; S-type cells are often prone to develop drug-resistance. [9]. An effective therapy for children with high-risk neuroblastoma (HR NBL) remains one of the greatest challenges for pediatric oncologists. Over the past two decades, numerous attempts have been made to improve outcomes of those patients by delivering intensive platinum cytostatics based on induction therapy [10]. Unfortunately, intensive chemotherapy, which is usually used for treatment of HR NBL, can cause genetic and expression changes in NBL cells. These cellular changes can cause development of drug-resistance to the drugs used, thus ultimately making the chemotherapy ineffective. Drug-resistance has been described as common in children suffering from NBL, particularly in those whose tumors display amplification and high-level expression of the *MYCN* oncogene [11]. High level MYCN expression is associated with unfavorable outcomes and a poor prognosis in tumors with amplified *MYCN* [12]. However, high level mRNA MYCN

expression in NBL lacking *MYCN* amplification seems to be associated with favorable outcomes [13]. High levels of MYCN mRNA in NBL with *MYCN* amplification may be explained by the fact that copies of the amplified gene are actively transcribed [14,15]. Overexpression of the *MYC* genes family generally induces unrestricted cell proliferation, inhibition of differentiation, cell growth, angiogenesis, reduced cell adhesion, metastasis, and genomic instability [16], while MYCN overexpression induces the malignant phenotype of NBL cells [14]. MYCN protein has been observed to induce apoptosis in different cells, including NBL and cells with apoptosis induced by cytostatics. NBL cells with *MYCN* amplification (and increased gene expression) can resist treatment only when there is additional dysfunction in the apoptotic pathways, such as caspase deficiency [17]. MYCN protein can also contribute in acquiring the drug-resistance phenotype of cancer cells by modulating the regulation of a specific set of ATP-binding cassette transporter genes to mediate the efflux of chemotherapeutic agents from cancer cells [18].

The aim of our study was to examine *MYCN* gene expression in different NBL cell lines, including N and S types, following a wide range (100 – 3000 ng/ml) of different cisplatin (CDDP) doses. An additional novel aspect of the study is a comparison of MYCN gene expression and copies of *MYCN* gene in NBL cells, sensitive or resistant towards CDDP, following single CDDP treatment (as above). *MYCN* gene copy numbers and its corresponding expression attracted our particular attention since this gene has become a frequent therapeutic target of NBL at present [19-21].

## 2. Materials and methods

### 2.1. Characteristics of the investigated cell lines

UKF-NB-3 and UKF-NB-4 cell lines with *MYCN* amplification (Fig. 1A and 1C) were established from bone marrow metastases of two patients with HR NBL. The UKF-NB-4 line that was established from recurrent disease already possessed the intrinsic multidrug resistance phenotype including 7q21 gain [22]. The lines were kindly provided by Prof. J. Cinatl Jr. (Institute for Medical Virology, Hospital of the Johann Wolfgang Goethe University, Frankfurt am Main, Germany). Cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal calf serum (PAA Laboratories, Pasching, Austria). The SK-N-AS cell line was derived from bone marrow metastasis of a female patient with HR NBL. This cell line, which has normal diploid *MYCN* status (Fig. 1E), was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was cultivated according to the manufacturer's instructions.

The CDDP-resistant counterparts, designated as UKF-NB-3$^{CDDP}$ and UKF-NB-4$^{CDDP}$, were also kindly provided by Prof. J. Cinatl Jr. SK-N-AS$^{CDDP}$ was prepared in our laboratory by incubation of parental SK-N-AS with increasing concentrations of CDDP either for 14 or 24 months. Solutions of CDDP (EBEWE Pharma Ges.m.b.H. Nfg. KG, Unterach, Austria) were prepared according to the manufacturer's instructions. CDDP-resistant cell lines were cultivated in a medium containing 1 μg/ml of CDDP.

### 2.2. Subsequent CDDP treatment

Finally, all above characterized cells were exposed to 100; 1000; 2000 and 3000 ng/ml CDDP 24 hours after seeding. After the next 24 hours, cells were harvested by trypsinisation, washed in sterile phosphate buffered saline and frozen with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at -80°C until RNA isolation.

### 2.3. MTT test

The sensitivity of cell lines to CDDP was determined by using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test [23,24]. The $IC_{50}$ was assessed using MTT dye reduction assay as described in a previous report [25]. The $IC_{50}$ values were calculated from at least 3 independent experiments using linear regression of the dose-log response curves using SOFTmax Pro microplate data software (Molecular Devices, CA, USA).

### 2.4. Fluorescent in situ hybridization (FISH) and karyotype

FISH analyses were performed by using an ON *MYCN* (2p24) / LAF (Kreatech Biotechnology B. V., Amsterdam, The Netherlands) probe, in accordance with the manufacturer´s instructions. The use of a 2p specific probe simultaneously with 2q signals is recommended by the International Neuroblastoma Risk Group Biology Committee [20]. Slides were scored with a Olympus AX70 (Olympus Optical Co. Ltd, Japan) fluorescence microscope with a CCD camera (Jenoptik, Jena, Germany). Pictures of the interphase nuclei were digitally captured by an appropriate software program (Metasystems, Altlussheim, Germany). Two hundred nuclei were investigated from each cell line as recommend for evaluation by FISH analyses [26].

For karyotype analysis, exponentially growing cells cultivated in a medium without CDDP were harvested with trypsin, treated with hypotonic solution, and fixed with 3:1 methanol/acetic acid. Slides were banded using standard trypsin-Giemsa banding (GTG-banding) or were used for metaphase FISH analysis.

### 2.5. RNA isolation and real-time RT-PCR

Total RNA was extracted from all cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of isolated RNA was verified by horizontal agarose gel electrophoresis and RNA quantity was measured using a Biomate 3 UV – Vis Spectrophotomether (Thermo Scientific, Waltham, MA). Isolated RNA (1 μg) was reverse transcribed to cDNA using random hexamers and a Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time RT PCR was assessed using *MYCN* and Beta-2-microglobulin primers and probe kits from Generi-Biotech (Generi-Biotech, Hradec Kralove, Czech Republic) and TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Results were expressed using the Relative Expression Software Tool (REST) [27] employing Beta-2-microglobulin as the reference gene as described earlier.

### 2.6. Statistical analysis

All numerical data were presented as mean ± standard deviation and analyzed statistically using the Student's t-test; mRNA expression results were analyzed using REST software [27].

# 3. Results:

## 3.1. The sensitivity of cell lines to CDDP

The sensitivity of cell lines to CDDP was verified using the MTT test. The resistant cell lines showed at least a two fold increase in resistance to CDDP measured as $IC_{50}$. The data presented in Table 1 indicate UKF-NB-3 line as the most sensitive and SK-N-AS$^{CDDP}$ and UKF-NB-4$^{CDDP}$ as the most resistant towards CDDP.

## 3.2. CDDP causes change in the number of *MYCN* copies

All tested NBL cell lines, the SK-N-AS, UKF-NB-3, UKF-NB-4 and their CDDP-resistant counterparts, were investigated for *MYCN* status using FISH. We observed *MYCN* amplification in all 200 investigated nuclei of UKF-NB-3, UKF-NB-4 and their CDDP-resistant counterparts (Fig. 1A-D). UKF-NB-3 and UKF-NB-4 amplified *MYCN* in the form of HSRs. UKF-NB-4$^{CDDP}$ cells had apparently fewer *MYCN* copies than UKF-NB-4 cells, as documented in Figure 1 C,D. Parental UKF-NB-4 cells had on average 62 excessive *MYCN* gene copies per nucleus, whereas UKF-NB-4$^{CDDP}$ cells lost on average 17 *MYCN* gene copies. In the UKF-NB-3 cell line, the aggregation of *MYCN* gene copies in the nucleus as clumps precluded a copy number counting with the exception of UKF-NB-3$^{CDDP}$ which had apparently less *MYCN* copies (24 excessive *MYCN* gene copies), as illustrated in Figure 1 A,B.

The *MYCN*-nonamplified SK-N-AS cell line showed normal diploid *MYCN* status in the majority of cells (Fig. 2A). After short-term (14 months) and long-term (24 months) cultivation with CDDP, SK-N-AS$^{CDDP}$ cells were examined again for the *MYCN* gene status and for the ploidy of chromosome 2. The CDDP cultivation resulted in a substantial 2p gain, since the SK-N-AS$^{CDDP}$ cells had 60% and 77% of cells with three 2p after short-term and long-term cultivation, respectively (Fig. 1G and 2A). On the contrary, in the SK-N-AS only 3.5% of cells were observed with three 2p (Fig. 2A). With regards to the *MYCN*-amplified cell lines, UKF-NB-3 and UKF-NB-3$^{CDDP}$ exhibited two signals for chromosome 2, while UKF-NB-4 cells had four signals and UKF-NB-4$^{CDDP}$ three signals for this chromosome (Fig. 1A-D).

Metaphase FISH analyses of SK-N-AS cells revealed a predominant occurrence of cells with two chromosomes 2 in mitotic cells. On the other hand, in SK-N-AS$^{CDDP}$ cells treated with CDDP, a substantial increase of cells with 2p gain had been recorded. We also observed cells with tetraploidy or aneuploidy, but their proportion was rather minor (Fig. 2B).

Above numeric changes in 2p may suggest the selection advantage of cells in the presence of CDDP.

### 3.3. MYCN expression levels in CDDP-sensitive and resistant NBL cell lines

Both *MYCN*-amplified UKF-NB-3 and UKF-NB-4 cell lines had significantly higher MYCN mRNA expression as compared to the *MYCN* non-amplified line SK-N-AS (14.8 respective 13.4-folds higher in 2-log scale, $p< 0.001$) (Fig. 3). SK-N-AS$^{CDDP}$ had MYCN expression 3.8-fold higher ($p< 0.01$) than SK-N-AS. We did not find any distinguishable change in MYCN expression between sensitive UKF-NB-3 and its CDDP resistant counterpart (Fig. 3), but UKF-NB-4$^{CDDP}$ had MYCN expression 1.6-fold higher ($p< 0.05$) in comparison to sensitive UKF-NB-4. There were no significant differences in MYCN expression between UKF-NB-3 and UKF-NB-4 nor between UKF-NB-3$^{CDDP}$ and UKF-NB-4$^{CDDP}$ cell lines. Regarding CDDP-resistant cell lines, the highest MYCN expression in comparison with SK-N-AS$^{CDDP}$ was found in UKF-NB-4$^{CDDP}$ (11.3-fold higher; $p< 0.01$), followed by UKF-NB-3$^{CDDP}$ (10.8-fold higher; $p< 0.01$).

### 3.4. MYCN expression levels in CDDP-sensitive and resistant NBL cells following short-term CDDP treatment

Sensitive UKF-NB-4 showed a significant decrease of MYCN expression after cultivation with CDDP concentrations of 1000 ng/ml and higher ($p=0.001$). All CDDP-resistant cells showed significant up-regulation of MYCN expression ($p=0.001$) in comparison with the sensitive cells, however, a single CDDP dose did not significantly modulate MYCN expression levels in resistant lines (Fig. 4A).

In the sensitive SK-N-AS we did not observe any significant changes in MYCN expression after cultivation with any CDDP dose ($p>0.3$). SK-N-AS$^{CDDP}$ cell line showed significant increase in MYCN expression relative to the control maternal line independently on CDDP presence or absence in the medium ($p<0.05$).

## 4. Discussion

In the present study, 6 NBL cell lines (4 with *MYCN* amplification and 2 cell lines without *MYCN* amplification) were analyzed. The cell line SK-N-AS$^{CDDP}$ (without *MYCN* amplification), which may serve as a model for the development of drug-resistant NBL, has been established and characterized in our laboratory. In the context of CDDP-based NBL therapy, we investigated the number of *MYCN* gene copies and mRNA MYCN expression. In the SK-N-AS cells and SK-N-AS$^{CDDP}$ cells we found a clear link between the number of *MYCN* gene copies and their expression. This finding is in agreement with the study of Valent et al, who found higher MYCN expression in three of four NBLs with 2p/*MYCN* gain in comparison with SK-N-SH cell line that has 1 *MYCN* copy in the haploid genome [28]. The cells with high-level *MYCN* amplification (UKF-NB-3, UKF-NB-4 and their CDDP-resistant counterparts) exhibit several-fold higher mRNA *MYCN* expression than those without *MYCN* amplification. But further changes in *MYCN* copies number in cells with high-level amplification were not accompanied by corresponding changes in mRNA expression. This phenomenon may be explained by the fact that not all *MYCN* copies could be equally and actively expressed. In this context, Schwab et al. described NBL cell lines in which the amounts of MYCN mRNA were not proportional to the exact number of gene copies [29]. On the other hand, in the case of NBL cell lines with normal number of *MYCN* copies and in NBL cell lines with one extra *MYCN* copy, the difference in MYCN expression was significant. The biological and clinical characteristics of 2p/*MYCN* gain have not been so far as clearly defined as *MYCN* amplification [30]. Approximately 10% of diagnosed NBLs contain *MYCN* gain [30,31]. Patients with *MYCN*-gain showed a significantly higher age at the diagnosis and were according some studies associated with an advanced stage as compared to the *MYCN*-normal [30,31]. In contrast to the study of Spitz et al., who reported that NBLs with *MYCN* gain displayed no increase in MYCN mRNA expression [31], and in agreement with Valent et al [28] who found in majority of NBLs with 2p/*MYCN* gain increased expression, we found a tendency of *MYCN* gain and higher MYCN expression in SK-N-AS$^{CDDP}$ cells.

Our results showed that all tested CDDP-resistant cell lines had higher (or at least equal) MYCN expression than their parental cells. We hypothesize that this overexpression may participate in the drug-resistance caused by long-term cultivation with CDDP. Decreases in the *MYCN* copy number after cultivation with CDDP in the *MYCN*-amplified UKF-NB-4 cell line might be explained by the expulsion of excessively amplified *MYCN* copies from

nuclei [7,32] or by selection of cells with lower *MYCN* copies. Long-term cultivation with CDDP leads to development of CDDP-resistance connected with decreased *MYCN* copy numbers in comparison to sensitive NBL cells [7,33]. However, decreases in the level of *MYCN* amplification could paradoxically be accompanied by higher MYCN expression [34,35]. This could explain why the decrease or increase in mRNA, caused by amplification, is not fully proportional to the number of gene copies, but might be influenced by other factors as well [1].

In addition to clinical (clinical stage, age), biochemical (LDH levels) and genetic (*MYCN* amplification, 1p36 deletion) prognostic parameters, the expressions of several genes have also been reported to predict the outcome of NBL. Overexpressed genes encoding tyrosine kinase receptors (TrkA and EPHB6) and cell surface molecules (CD44, EFNB2, and EFNB3) have been described as favorable markers [13], but the significance of *MYCN* expression remains controversial since survival of children suffering from NBL does not correlate with its expression [13,36,37]. A high expression of MYCN in NBL cells may be associated with both a favorable (cells without *MYCN* amplification) and adverse prognosis (cells with amplified *MYCN*) [12]. Similarly, MYC overexpression without amplification has also been linked with a more favorable prognosis in patients with breast [38] and colorectal cancers [39]. Better outcomes in patients with tumors that overexpress *MYC* or *MYCN* are caused either by increased apoptosis or by enhancement of drug-sensitivity evoked by higher proliferation [37]. Clinical observation has also been confirmed in *in vitro* experiments such as a transfection of cells with a vector containing *MYCN* which significantly decreased the viability of NBL cells without *MYCN* amplification. MYCN protein induced apoptosis and enhanced expressions of genes that were prognostically favorable [13]. *In vitro* studies with NBL cell line with a controlled MYCN expression system showed that NBL cells having induced MYCN expression have higher cytostatically induced apoptosis than NBL cells without induction of MYCN expression. Cytostatic drugs with various mechanisms of action including CDDP were tested. All tested compounds were more effective in cells overexpressing MYCN, except for betulinic acid, which induces apoptosis by direct effects on mitochondria [40]. The above-mentioned results seem to differ from our findings, reporting equal or increased MYCN expression in CDDP-resistant cells. An explanation could be that the induction of multiple changes in the genome of resistant cells indicates that cytostatics induce drug-resistance through multiple mechanisms [22,41]. It is possible that NBL cells without amplification of the *MYCN* used in the above mentioned study [40] have normally functioning apoptotic pathways, but in relation to drug-resistance, there is a defect in one of

these pathways, possibly due to the increased MYCN expression. Van Noesel et al. analyzed a panel of NBL cell lines with *MYCN* amplification and showed that *MYCN* induces both caspase-8 (CASP8) and caspase-9 (CASP9) mediated apoptosis. The authors concluded that an epigenetic down-regulation of CASP8 in NBLs is unlikely to be induced by overexpression of *MYCN*. Additional defects in apoptosis may be involved in the CASP9 route to apoptosis. It appears that defects in apoptotic routes in NBL tumors do have a neutralizing effect on *MYCN* [42].

As an *in vitro* model of drug-resistance [24], we have chosen CDDP-resistant sublines prepared by incubation of parental NBL cells with increasing concentrations of CDDP. We found that NBL cells with *MYCN* amplification, when subjected to long-term cultivation with CDDP, showed decreased numbers of *MYCN* copies. On the other hand, the NBL cell line, with mainly diploid status of the *MYCN* gene, had increased numbers of cells with 2p gain and increased MYCN expression. Wasenius et al., described both deletion and gain of 2p in CDDP-resistant ovary cancer cell lines [43] but our finding of 2p gain in NBL in connection with drug-resistance was described for the first time. We studied MYCN expression in sensitive and CDDP-resistant NBL cells after exposure to CDDP as an *in vitro* model of chemotherapy. Our findings contribute to the understanding of the influence of chemotherapy on MYCN expression in NBL cell lines. This is of particular importance since targeting the *MYCN* gene might be new therapeutic alternative for NBL [19-21].

## Conflict of interest

The authors have no conflicts of interest.

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## Figure Legends

### Fig. 1.

**_MYCN_ gene in tested NBL cell lines.**

FISH displays *MYCN* amplification (red signals) in nuclei of the UKF-NB-3 and UKF-NB-4 (Fig. 1A, C, respectively) and their CDDP-resistant counterparts (Fig. 1B, D). The SK-N-AS cell line has two copies of *MYCN* gene and two chromosomes 2 (green signals) (Fig. 1E). In the CDDP-resistant SK-N-AS cells after short term treatment with CDDP (14 months), the nuclei exhibits an extra copy of the *MYCN* gene (red signals) representing either 2p gain or *MYCN* gain or both (Fig. 1F). Mitotic chromosomes of the SK-N-AS$^{CDDP}$ after long term treatment (24 months) with CDDP showed an extra copy of the short arm of chromosome 2 containing the *MYCN* gene (red dots) and an extra copy of reference *LAF* gene (green dots) (Fig. 1G). FISH analyses were performed using an ON *MYCN* (2p24) / LAF (Kreatech Biotechnology B. V., Amsterdam, The Netherlands) probes. Magnified 100x

### Fig. 2a.

**Ploidy of chromosome 2 and percentage of cells with _MYCN_ gain in the SK-N-AS cells during cultivation with CDDP**

For the assessment 200 interphase nuclei of the tested line were scored. Three signals corresponding to 2p24 were evaluated as 2p gain, more than 3 signals of 2p24 in relation to the *LAF* gene signal were evaluated as *MYCN* gain. Ploidy of chromosome 2 was scored using an ON *MYCN* (2p24) / LAF (Kreatech Biotechnology B. V., Amsterdam, The Netherlands) probe.

### Fig. 2b.

**Number of chromosome 2 in mitotic SK-N-AS cells**

In each mitosis the number of chromosome 2 was scored and expressed as a percentage of cells with normal and abnormal number of chromosome 2. The type of chromosome 2 gain/loss is also indicated. Investigated cells were prepared by typical cytogenetic preparation using colcemid for metaphase observation. Analyses were performed using an ON *MYCN* (2p24) / LAF (Kreatech Biotechnology B. V., Amsterdam, The Netherlands) probes.

**Fig. 3.**

**MYCN expression in NBL cell lines and their CDDP-resistant counterparts**

The data are expressed as relative expression levels following the comparison to the reference NBL line SK-N-AS. Expression analysis of reference gene Beta-2-microglobulin was carried out parallel with all experiments and mRNA levels were not different in any expression analysis for particular cell line.

**Fig. 4a.**

**MYCN expression in UKF-NB-4 and UKF-NB-4$^{CDDP}$ cells after treatment with CDDP.**

Sensitive-maternal UKF-NB-4 cells are represented by black bars, CDDP-resistant UKF-NB-4 cells are represented by gray bars. The dose of CDDP is depicted on x axis. Untreated CDDP-sensitive UKF-NB-4 cells were used as a reference value. Experiment was repeated three-times with concordant outcomes.

**Fig. 4b.**

**MYCN expression in SK-N-AS and SK-N-AS$^{CDDP}$ cells after treatment with CDDP.**

Sensitive-maternal, *MYCN* non-amplified, SK-N-AS cells are represented by black bars, CDDP-resistant SK-N-AS cells are represented by gray bars. The dose of CDDP is depicted on x axis. Untreated CDDP-sensitive SK-N-AS cells were used as a reference value. Experiment was repeated twice.

**Table 1 – Level of drug-resistance of tested NBL cell lines expressed as $IC_{50}$**

| Cell line | NBL type | $IC_{50}$ (μM) CDDP |
|---|---|---|
| SK-N-AS | S | 3.71 ± 0.67 |
| SK-N-AS$^{CDDP}$ | S | 11.68 ± 2.84 |
| UKF-NB-3 | N | 0.99 ± 0.01 |
| UKF-NB-3$^{CDDP}$ | N | 7.68 ± 1.37 |
| UKF-NB-4 | S | 4.60 ± 0.2 |
| UKF-NB-4$^{CDDP}$ | S | 11.33 ± 1.12 |

$IC_{50}$ values were calculated from the linear regression of the dose-log response curves after 96 hours exposure to CDDP, determined by the MTT assay. Values are mean ± SD of at least 3 independent experiments. S, S-type of NBL; N, N-type of NBL.

Figure1
Click here to download high resolution image

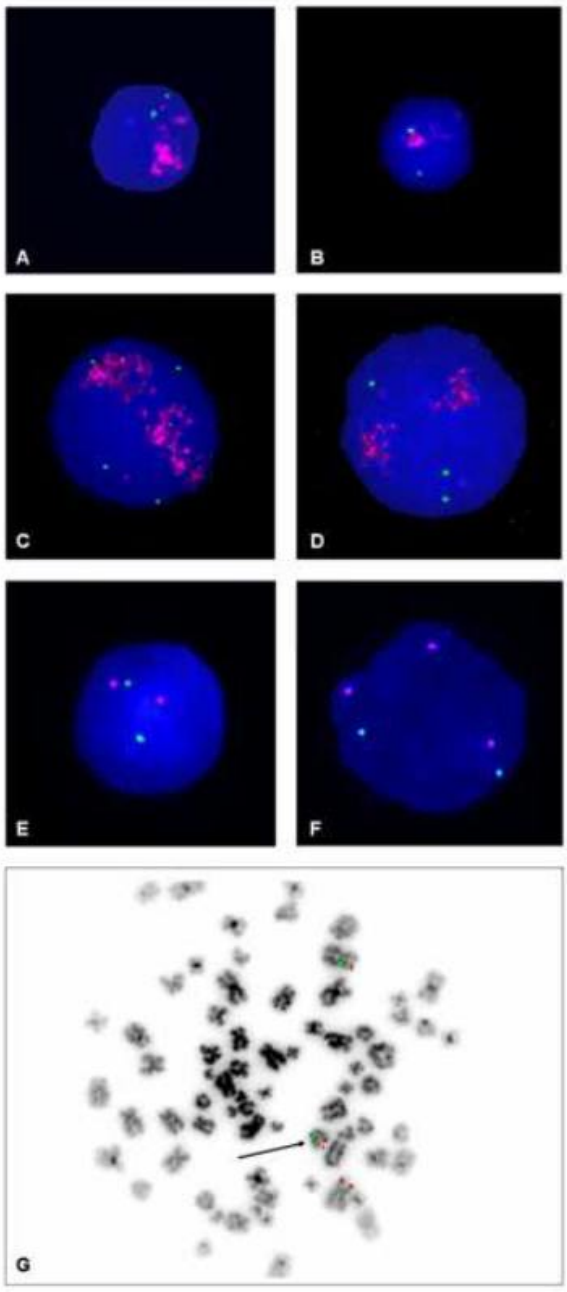


Figure2

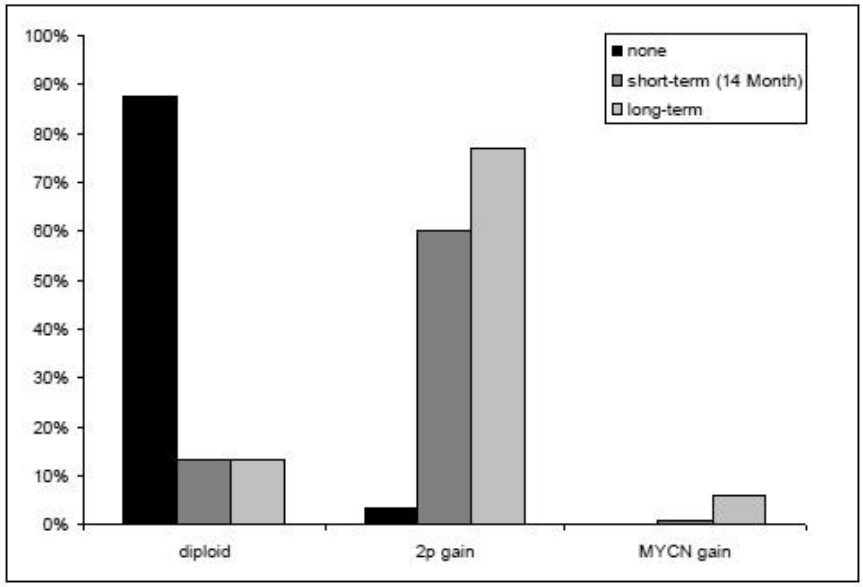



Figure 2A

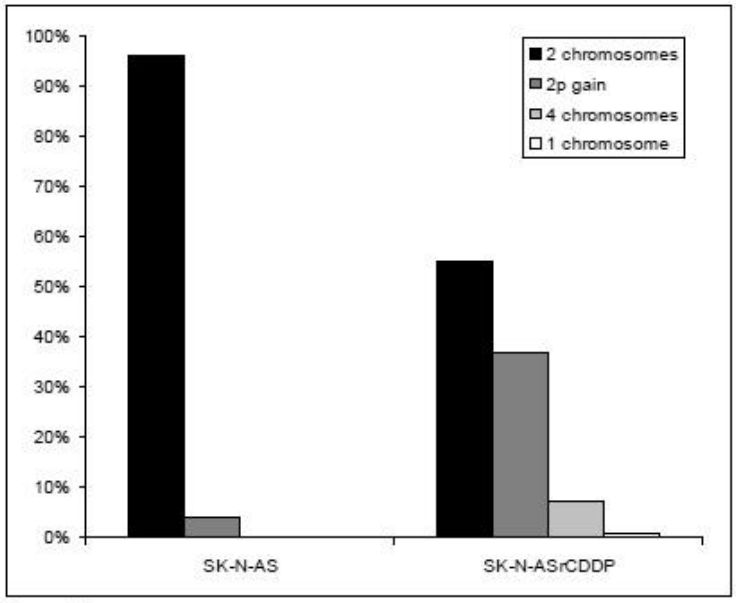



Figure 2B

Figure3

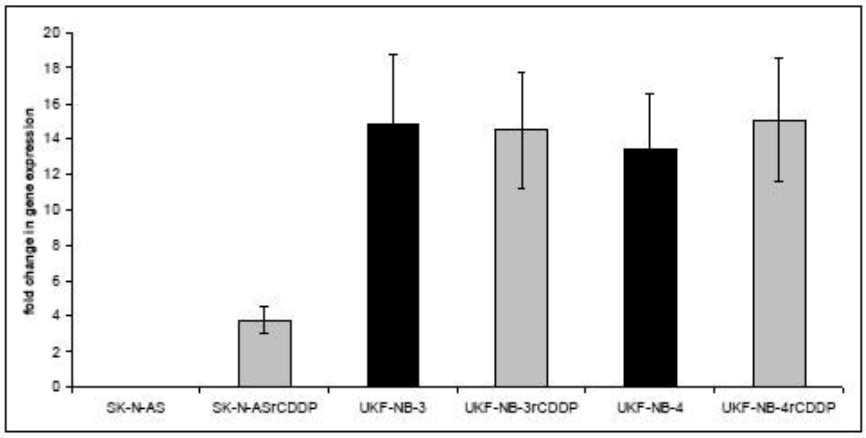



Figure 3

**Figure4**

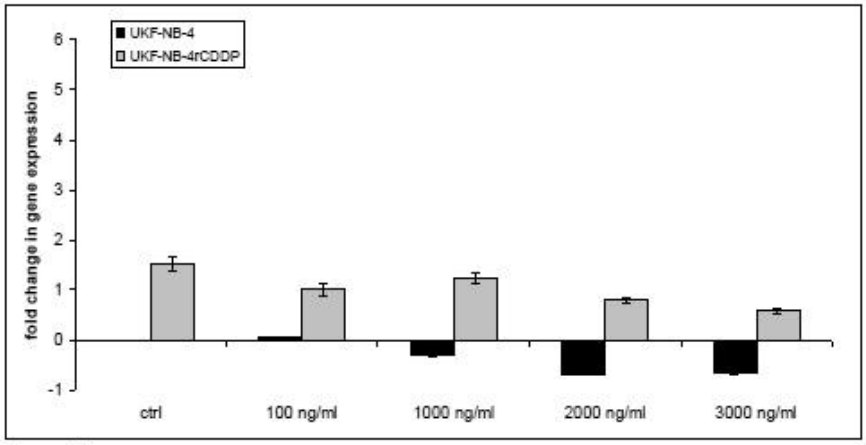



Figure 4A

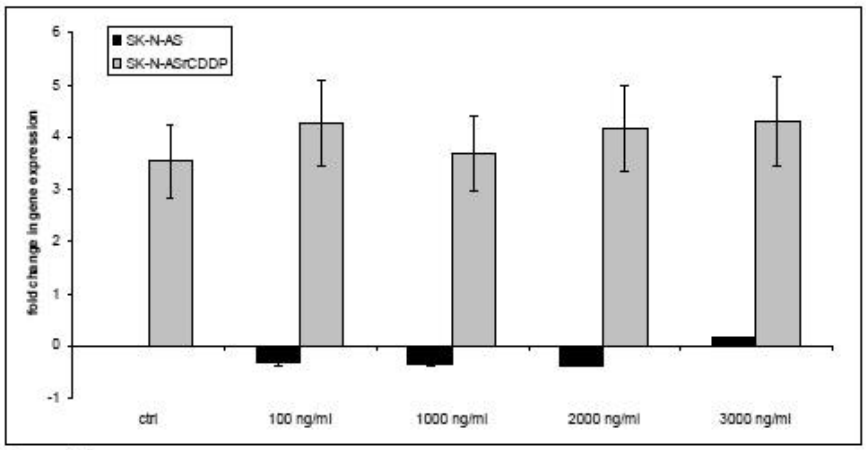



Figure 4B

# 3 Diskuze

## *3.1 Genetické změny Ewingova sarkomu a feochromocytomu u dětí (Práce I a II)*

### 3.1.1 Genetika Ewingova sarkomu a její využití v léčbě

Ewingovy sarkomy, PNET a nediferencované sarkomy jsme na Klinice dětské hematologie a onkologie UK 2. LF a FN Motol (KDHO) vyšetřovali metodou CGH a reverzně transkripční polymerázovou řetězovou reakcí (Reverse Transcription Polymerase Chain Reaction, RT PCR) pro průkaz specifických translokací. U dvou nádorů Ewingova sarkomu hrudní stěny jsme nalezli specifickou translokaci (11;22) a zmnožení 8. chromozómu. Zmnožení 8. chomozómu je považováno za sekundární změnu provázející horší prognózu [30]. U pacienta s PNETem žebra byla detekována specifická translokace t(11;22), ztráta 16q a zmnožení 1q a 9q32-qter, které mohou odpovídat nebalancované translokaci (1;16). Dva ze tří nediferencovaných sarkomů, u kterých nebyla zjištěna translokace t(11;22), měli zmnožení 8. chromozómu. Tato změna tedy není specifická pro Ewingovy sarkomy a je nejspíše sekundární. U pacientů léčených na KDHO s Ewingovými sarkomy jsme nalezli metodou CGH změny popsané v literatuře - zmnožení 8. chromozómu, deleci 16q se zmnožením 1q [31]. Přítomnost sekundárních změn může být při použití technik s vysokým rozlišením, jako je například aCGH, používáno k hodnocení prognózy onemocnění a volbě optimální léčby. V současné době se zvažuje použití genotypického profilování a RNA interference [32] k identifikaci nových cílů léčby Ewingových sarkomů. Například ve studii Arora a spolupracovníků byly nalezeny dvě kinázy, STK10 a TNK2, které mají potenciál stát se terapeutickým cílem nových léčiv [33]. Stejně tak downregulace EWS/Fli-1, produktu výše zmíněné translokace t(11;22) (q24;q12), pomocí RNA interference může být novým a účinným přístupem v léčbě Ewingova sarkomu. Bylo zjištěno, že utlumení EWS/Fli-1 snižuje i expresi proteinu c-Myc, které inhibuje proliferaci nádorových buněk [34]. Proto průkaz těchto změn může posloužit k predikci efektu cílené léčby.

### 3.1.2 Genetické změny dětských feochromocytomů

Maligní feochromocytomy jsou u dětí velmi vzácné. Faktory signalizující příznivou prognózu u dětských feochromocytomů jsou vyšší věk, nepřítomnost genetických syndromů v rodinné anamnéze, diploidní stav. Rozlišení benigního a maligního feochromocytomu není možné na základě klinických, biochemických ani histopatologických vlastností [35]. Údaje o genetických změnách, které mohou určit biologickou povahu feochromocytomů jsou dosud nedostatečné, ale některé chromozomální změny (ztráta oblasti 11q22 – qter a delece 6q) a aneuploidie jsou nalézány častěji u zhoubných nádorů.

U všech čtyř dětí s feochromocytomem jsme detekovali deleci postihující 11p (třikrát pouze 11p a jednou celého 11. chromozómu). Delece 1p a části 3p respektive celého 3. chromozómu byla nalezena u dvou pacientů. Ztráty 1p a 3q patří k nejčastějším změnám nacházeným u feochromocytomů dospělých [36]. Různé změny v oblasti 11. chromozómu (del 11p nebo 11q, zmnožení 11q) a del 17p jsou udávány převážně u maligních forem feochromocytomů dospělých [10]. My jsme nalezli deleci 11p u tří a celého 11. chromozómu u čtvrtého z našich pacientů a u jednoho navíc deleci 17p přestože neměli známky malignity (metastázy). Otázkou je, zda metastázy nebyly přítomny, protože onemocnění bylo zachyceno relativně včas nebo se jednalo o benigní formu. Druhou možností může být odlišnost genetických změn u feochromocytomů dětí a dospělých. Řada nádorů, které jsou typické pro dospělý věk, ale vzácněji se vyskytnou i u dětí, má v obou těchto věkových skupinách odlišnosti biologické, klinické i genetické. Největší počet genetických změn byl nalezen u chlapce s mutací genu VHL a oboustranným feochromocytomem. Pokud je nám známo, tato námi prokázaná mutace dosud nebyla u von Hippel Lindauova syndromu popsaná.

U dětských feochromocytomů byla prokázána souvislost mezi lokalizací nádoru, mutovaným genem a chromozomálními změnami. Zajímavá je častější delece v oblasti 11. chromozómu. V této oblasti se nachází řada genů, které by mohly mít vliv na inicializaci nádorového onemocnění [37]. Pro vznik feochromocytomů bude pravděpodobně nutná více než jedna genetická změna a tyto změny jsou často odlišné u paragangliomů a feochromocytomů.

## *3.2 Mechanismy rezistence neuroblastomových buněk k ellipticinu (Práce III)*

### 3.2.1 Ellipticin a jeho léčebné využití

Ellipticin (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) je alkaloid, izolovaný z rostlin čeledi *Apocyanaceae* (jako jsou (*Ochrosia borbonica*, *Excavativa cocinea*). Poprvé byl izolován v roce 1959 z listů stále zeleného stromu *Ochrosia elliptica* rostoucího v Oceánii. Jeho protinadorová aktivita je popsána experimentálně [38] i v klinických studiích [39]. Od 70. let 20. století byly polárnější deriváty ellipticinu (9-methoxyellipticin a 2-methyl-9-hydroxyellipticin) ve formě acetátu (NMHE) úspěšně používány ve Francii k léčbě karcinomu prsu s kostními metastázemi, akutní myeloblastické leukémie a anaplastického karcinomu štítné žlázy [39]. Ellipticin také inhibuje retrovirovou integrázu viru HIV a je proto zkoumán jako možný lék u AIDS [40]. Za hlavní cytotoxický a protinadorový účinek ellipticinu a jeho derivátů je považována interkalace do DNA a inhibice topoizomerázy II alfa (TOP2A) [41, 42]. Dále bylo zjištěno, že ellipticin je schopen aktivovat mutantní p53 a selektivně inhibuje fosforylaci proteinu p53, což by mohlo mít význam u zhoubných nádorů s mutací p53 [43]. Rovněž narušuje energetickou rovnováhu buněk odpřahováním oxidativní fosforylace v mitochondriích [44] a zabraňuje proliferaci buněk regulací exprese cyklinu B1 a Cdc2 a fosforylací Cdc2 [45]. Haag a spolupracovníci popsali ellipticinem zprostředkovanou apoptózu indukovanou stresem endoplasmatického retikula [46]. Důvodem zájmu o ellipticin je jeho vysoká účinnost proti nádorovým buňkám již v koncentracích od 0,1 – 1 μM [47] a jeho relativně nízké toxické účinky. Limitující toxicitou je xerostomie (asialie), která může vyvolávat další nežádoucí účinky jako jsou monilázy nebo anorexie. S výjimkou nefrotoxicity, která je svým mechanismem vzniku podobná nefrotoxicitě cisplatiny, jsou další vedlejší nežádoucí účinky ellipticinu minimální. Hematologická toxicita, která limituje dávkování většiny cytostatik, je prakticky nulová [48]. Ellipticin a jeho 9-hydroxyderivát vykazují mutagenní aktivitu vůči kmenům Escherichia coli, Neurospora crassa, Salmonella typhimurium i vůči buňkám savčím [49]. Při léčbě byla pozorovaná individuální variabilita v odpovědi pacientů na podané léčivo. Vysvětlením může být rozdílná individuální výbava enzymy důležitými pro biotransformaci ellipticinu (cytochromy P450, cyklooxygenáza,

myeloperoxidáza). Tyto enzymy aktivují léčivo na terapeuticky účinnější deriváty, které vytvářejí s DNA kovalentní adukty poškozující nádorové buňky [50].

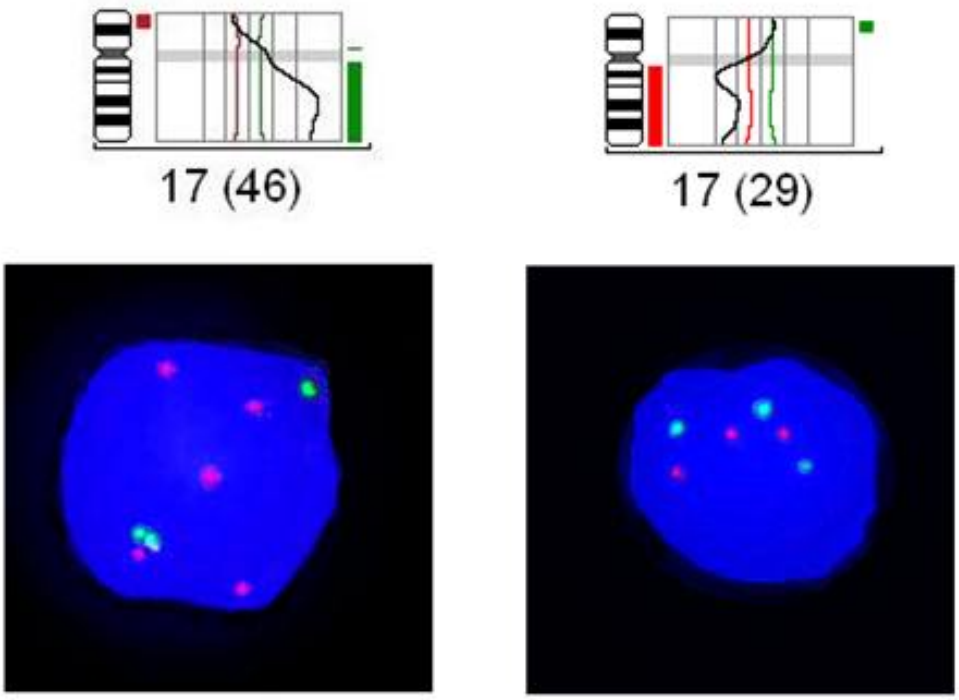
### 3.2.2 Genetické změny vyvolané kultivací s ellipticinem a jejich srovnání s ostatními cytostatiky

Cytogenetické změny nádorových buněčných linií vzniklé v průběhu pasážování buněk je obtížné rozlišit od cytogenetických změn indukovaných cytostatikem [51]. Buněčné linie odvozené od solidních nádorů vykazují množství cytogenetických změn, stejně tak od nich odvozené chemorezistetní dceřiné linie [52]. Odlišení změn, které jsou charakteristické pro parentální buněčnou linii a nově vzniklé změny u dceřiné linie umožňuje použití modifikace CGH současné hybridizace různě fluorescenčně značených DNA z mateřské a rezistentní linie (Obrázek č. 5). Tímto postupem se zachytí pouze změny vzniklé selekčním procesem [53]. Vyšetření pomocí CGH a doplněné o analýzu mFISH a iFISH (Obrázek č. 5) detekuje jak balancované, tak nebalancované změny napříč genomem [54].

U chemorezistentních nádorových buněk je časté zmnožení nebo amplifikace oblasti 7q21, kde se nachází gen *MDR1* kódující P-glykoprotein [52]. U buněčné linie UKF-NB-4 připravené z chemorezistentího relapsu je zmnožena oblast 7q21. Po její dlouhodobé inkubaci v médiu s ellipticinem se naopak počet kopií 7q21 snižuje a rezistentní linie již žádné nadbytečné kopie nevykazuje. Analýza exprese na úrovni proteinu odhalila sníženou expresi P-glykoproteinu. Na rozdíl od linie rezistentní k doxorubicinu, která vykazuje 3,5 násobek nárůstu exprese, exprimuje linie rezistentní k ellipticinu P-glykoprotein v polovičním množství ve srovnání s parentální linií [54].

Krátké raménko 16. chromozómu s lokalizací genu *ABCC1* kódující jeden z ABC transportérů (multidrug resistence protein 1, MRP1, 16p13.1) bývá v neuroblastomových buňkách zmnoženo [55] a jeho souvislost s rezistencí k doxorubicinu je zdokumentována [56]. Obdobně bývá v neuroblastomových buňkách zmnožena oblast 12. chromozómu s lokalizací genu *LRP1* (low density lipoprotein receptor-related protein 1, 12q13-14) [55]. Žádná z těchto cytogenetických změn se v linii rezistentní k ellipticinu nevyskytuje. Některé změny exprese nemají podklad ve změnách genómu. Například snížení exprese CD56 (NCAM, neural cell adhesion molecule) nalezené v rezistentní linii k ellipticinu, které je popisováno i u linií

rezistentních k vinkristinu a doxorubicinu [57], není u linie rezistentní k ellipticinu provázeno zvýšením počtu kopií oblasti 11q23 (lokalizace genu pro NCAM) [54].




**Obrázek č. 5: Detekce změn u chemosenzitivní a její dceřiné linie rezistentní k ellipticinu.** Metodou CGH byl získán poměr intenzit fluorescence mezi senzitivní UKF-NB-4 (značena zeleně) a kontrolní DNA (značena červeně). Linie UKF-NB-4 vykazuje ztrátu oblasti 17p13 a zmnožení oblasti 17q. Rezistentní linie UKF-NB-4$^{ELLI}$ (značená zeleně) vykazuje zmnožení oblasti 17p13 a ztrátu dlouhého raménka 17. chromozomu (ve srovnání s UKF-NB-4 značenou červeně). Vyšetření iFISH sondami ON ERBB2, Her-2/neu (17q12) / SE 17 (KREATECH Diagnostics, The Netherlands), určí počty kopií u obou vyšetřovaných linií. Linie mají trizómii 17. chromozómu (centromérická sonda SE 17 značená zeleně), UKF-NB-4 má zmnoženou oblast 17q (5 červených signálů) zatímco linie UKF-NB-4$^{ELLI}$ ztratila nadbytečné kopie 17q (3 červené signály) a počet kopií odpovídá trizómii 17. chromozomu. Vyšetření iFISH koresponduje s CGH profilem.

## *3.3 Změny počtu kopií onkogenu MYCN a jeho exprese ovlivněné působením cytostatik v neuroblastomových buňkách (Práce V a VI)*

### 3.3.1 *MYCN*

Produktem genu *MYCN* (V-myc myelocytomatosis viral related oncogene, neuroblastoma derived) je jaderný fosfoprotein N-myc s vazebnou afinitou k DNA ovlivňující transkripci. N-myc se podílí na regulaci buněčného cyklu, na buněčném růstu, na programované buněčné smrti [58] a je nezbytný pro vývoj a diferenciaci neuroektodermu. Ve zdravých buňkách je N-myc exprimován pouze v embryonálních tkáních, v dospělých nenádorových buňkách je jeho exprese velmi nízká nebo zcela chybí [59].

### 3.3.2 Numerické změny onkogenu *MYCN* v průběhu *in vitro* chemoteparie

*MYCN* patří do *MYC* genové rodiny zahrnující *MYC*, *MYCN* a *MYCL*. *MYCN* byl prvním popsaným onkogenem jehož amplifikace byla nalezena v nádorech. Průkaz jeho amplifikace získal velký klinický význam díky vztahu s velmi špatnou prognózou u neuroblastomu [60, 61]. *MYCN* je lokalizován na 2. chromozómu v oblasti 2p24. V průběhu vzniku nádoru může docházet k mnohonásobné duplikaci genů, které nádorové buňce pomáhají v rychlejším dělení a mohou též způsobit odolnost buňky vůči účinkům cytostatik. Mechanismus vzniku amplifikace *MYCN* v neuroblastomu je stále neznámý, ale zdá se, že nejlépe odpovídá tzv. „replication - excision“ modelu [62]. Při kterém je DNA opakovaně replikovaná pouze v jedné chromozomální oblasti, nadbytečná DNA je následně vystřižena a přetrvává ve formě malých párových acentrických extrachromozomálních chromatinových tělísek, tzv. „double minutes“ (double-minute chromatin bodies). Tato extrachromozomální DNA může být integrována do jiného chromozómu v podobě intrachromozomalní homogenně se barvící oblasti (Homogenously Staining Regions, HSR) [63], kde může být dále amplifikována. „Double minutes“ představují nejčastější uspořádání amplifikovaného genu *MYCN* v buňkách neuroblastomu, ale v buněčných liniích odvozených od neuroblastomu je amplifikace *MYCN* ve formě „double minutes“ méně častá. Amplifikace *MYCN* představuje obvykle 50–100 kopií [64]. Prognostický význam

přesného počtu kopií amplifikovaného *MYCN* nebyl dosud prokázán, protože nádorové buňky obsahující nejméně čtyři kopie MYCN na jeden 2. chromozom, jsou vždy agresivní. Zmnožení počtu kopií genu *MYCN* klasifikujeme do dvou skupin. Gain představuje zmnožení kopií nepřesahující čtyřnásobek počtu referenčních signálů odpovídající kontrolní sondě na dlouhém raménku 2. chromozómu (2q signál) při vyšetření FISH. Gain může být podmíněn jak zmnožením samotného genu *MYCN,* tak celého krátkého raménka 2. chromozómu (2p gain), které je u neuroblastomu nezávislým prognostickým faktorem [5].

*MYCN* amplifikuje v časném stádiu vývoje nádoru a v dalším průběhu onemocnění se amplifikace zpravidla nemění. Přesto existují experimentální i klinické práce popisující početní změny *MYCN* v nádorových buňkách. Často se jedná o reakci buněk na chemoterapii [65]. Vypuzování amplifikovaných kopií genů bylo v minulosti popsáno pouze u amplifikace typu „double minutes“ [66, 67]. Ztráty nadbytečných amplifikovaných kopií genu *MYCN* ve formě double minutes byly pozorovány jak v buňkách získaných od pacientů, tak v buňkách z linií [68-70]. Nejpravděpodobnějším mechanismem ztrát kopií je formování tzv. mikrojader obsahujících nadbytečné kopie *MYCN*. Tato „mikrojádra“ jsou vypuzena do cytoplasmy, kde jsou degradována [67]. Spontánní vypuzování mikrojader je zpravidla pomalé, ale může být zrychleno působením cytostatik, zejména hydroxyurey. Bylo zjištěno, že úbytek amplifikovaných kopií snižuje tumorigenicitu nádorových buněk [66]. Vypuzování nadbytečných kopií *MYCN* z oblastí HSR při kultivaci s cytostatiky jsme jako první popsali až v roce 2010 [63]. Na druhé straně jsme také nalezli zvýšení počtu kopií *MYCN* u diploidní neuroblastomové linie jako následek *in vitro* chemoterapie, která navodila chemorezistenci (Procházka P., submitted 2012). Tento nález by mohl vysvětlit popsanou horší prognózu pacientů se zmnožením *MYCN* [71, 72]. Wasenius a kol. nalezli zmnožení *MYCN* v buněčných liniích odvozených od nádorů vaječníků s rezistencí k cisplatině [73], ale zmnožení *MYCN* u linie neuroblastomu při vzniku chemorezistence dosud není v literatuře uváděno. Vyšší exprese mRNA MYCN u neuroblastomové linie se zmnožením *MYCN* je v souladu s prací Valenta a kol. [74].

### 3.3.3 Význam onkogenu *MYCN* v chemorezistenci neuroblastomu

Efektivní terapie dětí s neuroblastomem vysokého rizika (HR NBL) zůstává jednou z největších výzev pro dětské onkology. Za dvě poslední desetiletí bylo

zavedeno mnoho nových léčebných postupů založených na indukční terapii platinovými cytostatiky [75]. Bohužel intenzivní chemoteparie může vyvolat genetické nebo expresní změny u neuroblastomových buněk. Tyto změny pak mohou způsobit vznik rezistence k použitým léčivům a chemoterapie se pak stává neúčinnou. Resistence nádorových buněk k platinovým cytostatikům je v současné době předmětem intenzivního zájmu [76-78]. Léková rezistence je častá u dětí s diagnostikovaným neuroblastomem zejména pokud neuroblastomové buňky obsahují amplifikovaný nebo zmnožený onkogen *MYCN* spojený s jeho vysokou expresí [79]. Vysoká exprese mRNA MYCN je asociována s nepříznivou prognózou u nádorů s amplifikací *MYCN* [2], ale v nádorech bez amplifikace *MYCN* naopak představuje známku příznivější prognózy [80]. Vysoká exprese MYCN v neuroblastomech s amplifikací *MYCN* může být vysvětlena faktem, že kopie amplifikovaných genů jsou aktivně přepisovány a zvýšený počet genových kopií způsobuje vyšší expresi [81, 82]. Zvýšená exprese genů z rodiny *MYC* navozuje rychlé buněčné dělení, proliferaci, inhibuje diferenciaci, stimuluje angiogenesi, snižuje buněčnou adhezi, zvyšuje genomickou nestabilitu a indukuje maligní vlastnosti neuroblastomových buněk [81, 83]. N-myc je zodpovědný za řízení apoptózy v různých buňkách zahrnujících buňky neuroblastomu a buňky s navozenou apoptózou pomocí cytostatik. Neuroblastomové buňky s amplifikací *MYCN,* a s tím spojenou zvýšenou expresí, mohou odolávat léčbě pouze tehdy, pokud mají porušené apoptotické dráhy, například downregulaci kaspáz [84]. N-myc může také přispívat ke vzniku chemorezistence nádorových buněk regulací exprese specifických genů kódujících ABC transportéry, které ovlivňují efflux cytostatik z nádorových buněk [85].

### 3.3.4 Terapeutické využití *MYCN*/N-myc

Chemorezistentní vlastnosti buněk neuroblastomu vysokého rizika představují závažný problém při použití klasické cytostatické léčby. Jak již bylo výše uvedeno, amplifikace *MYCN* a jeho zvýšená exprese způsobují agresivní chování neuroblastomových buněk spojené s ovlivněním řady důležitých buněčných procesů. Proto se *MYCN* a jeho protein N-myc stávají cílem nově vyvíjených terapeutických postupů [86]. V současné době se v léčbě neuroblastomu používá intenzivní chemoterapie, chirurgická resekce nádoru, myeloablativní chemoterapie s autologní transplantací, radioterapie, 13-cis retinová kyselina, v některých protokolech i systémová radioterapie $I^{131}$meta iodobenzyl guanidinem (MIBG) nebo monoklonální

protilátky proti disialogangliosidu GD2. V klinických studiích u neuroblastomů s amplifikací *MYCN* se nyní testuje použití např. temozolamidu, fenretinidu, inhibitorů proteazómu, nifurtimoxu, cixutumumabu [87]. Snížení exprese N-myc by mohlo obnovit genomickou stabilitu buněk a při odpovídající funkci proteinu p53 v kombinaci s vysokými dávkami cytostatik může zlepšit výsledky léčby neuroblastomu. Ke snížení exprese je možné použít RNA interferenci, která eliminuje nadměrnou expresi mRNA MYCN [88]. Z experimentálních výsledků vyplývá, že RNAi je spojena s aktivací kaspázy 3, upregulací p27 a downregulací Bcl-2 a MDM2. Snížení exprese MYCN navozené pomocí lentivirového vektoru v buňkách neuroblastomových linií IMR-32 a LAN-1 významně snižuje růst nádoru *in vitro* i *in vivo* [89]. Další možností je downregulace cílových proteinů, které N-myc ovlivňuje. Jako potenciálně vhodné cíle terapie se jeví Dickkopf3 (DKK3), MDM2 a SKP2, společně s ovlivněním signalizačních drah CDC42 a WNT [90]. Exprese tumor supresorového genu DKK3 se zvyšuje po deaktivaci *MYCN* v neuroblastomových liniích IMR-32 a SK-N-BE [91]. Použitím RNA interference proti MDM2 bylo zjištěno, že MDM2 pozitivně ovlivňuje expresi MYCN v neuroblastomových liniích LA1-55N (p53-null) a NB-1691 (p53 wild type) s amplifikací *MYCN* pomocí p53 nezávislé regulace jak na úrovni stabilizace mRNA, tak translace. Na druhou stranu v linii SHEP-Tet/21N, která nemá amplifikaci *MYCN,* RNA interference proti MDM2 expresi MYCN neovlivňuje [92]. Vysoká exprese regulátoru buněčného cyklu SKP2 má velký význam ve vývoji a progresi neuroblastomů bez ohledu na počet kopií genu *MYCN* [93]. Pokud je *MYCN* deregulován, dochází k aktivaci a overexpresi SKP2, což ovlivňuje buněčné dělení neuroblastomových buněk [94]. U linií SHEP-2, SHEP-21N a SK-N-AS byl popsán mechanismus inaktivace CDC42 pomocí RNA interference a naznačeny možnosti jeho využití v klinické praxi [95]. Již od devadesátých let minulého století, kdy byla aktivace WNT signalizace poprvé prokázána u melanomu a karcinomu tlustého střeva, se farmaceutický a biotechnologický průmysl začal zabývat vývojem efektivních inhibitorů WNT signalizace. Navzdory tomuto dlouhému vývoji stále ještě nebyly vyvinuta léčiva použitelná ke klinickým zkouškám [96].

# 4 Závěry

Tato práce se zabývá chemorezistencí dětských nádorů a detekcí cytogenetických změn které s ní souvisí. Průkaz těchto chromozomálních aberací může přispět k určení prognózy a predikce efektu léčby. Většinu výsledků jsme získali studiem neuroblastomu.

V první části této práce byla zpracována literární rešerše genetických změn u Ewingova sarkomu v souvislosti se zaváděním nových molekulárně cytogenetických metod jako je CGH a aCGH doplněná o vlastní výsledky. V druhé části práce byly metodami CGH a aCGH popsány cytogenetické změny u dětských feochromocytomů a paragangliomů. Byly identifikovány běžně popisované cytogenetické změny v chromozomových oblastech 1p, 3, 3p, 11, 11p. Přestože byli do studie zahrnuti pouze 4 pacienti, jedná se o jeden z největších souborů podrobně geneticky vyšetřených dětských feochromocytomů.

Stěžejní částí celé práce je studie, která navazuje na analýzu účinků ellipticinu na buňky neuroblastomu a rozšiřuje ji o poznatky týkající se chemorezistence k ellipticinu, léčivu využitelnému u neuroblastomu. V laboratoři biologie solidních nádorů Kliniky dětské hematologie a onkologie byla připravena dlouhodobou kultivací neuroblastomové linie UKF-NB-4 v mediu s cytostatikem dceřiná rezistentní linie. Porovnali jsme růstové charakteristiky *in vitro* i *in vivo* parentální (senzitivní) i rezistentní linie a detekovali cytogenetické změny na úrovni DNA, změny exprese mRNA a vybraných proteinů. Bylo zjištěno, že ABC transportéry se neuplatňují v chemorezistenci k ellipticinu, naopak významná je upregulace topoizomeráz a Bcl-2. Jako doplňkové mechanismy byly identifikovány upregulace enzymů oxidativní fosforylace, buněčného dýchání, V-ATPázy, aerobní respirace, spermine syntetázy v kombinaci s pomalejším růstem. Studie potvrzuje, že chemorezistence nádorových buněk nepředstavuje jeden mechanismus, ale komplex mechanismů, které se vzájemně doplňují. Z tohoto důvodu bude k jejich překonání nutný komplexní přístup.

Poslední část se týká nejdůležitějšího prognostického znaku u neuroblastomu - amplifikace onkogenu *MYCN*. V této práci byly detekovány změny v počtu kopií a exprese mRNA MYCN při krátkodobé nebo dlouhodobé *in vitro* léčbě. U linií IMR-32 a UKF-NB-4 jsme prokázali vypuzování amplifikovaných kopií z oblastí HSR

po účinku cytostatik cisplatiny, doxorubicinu, vikristinu a zvláště hydroxyurey. Vypuzování *MYCN* amplifikovaného ve formě double minutes je známe, ale jako první jsme prokázali vypuzování amplifikovaného genu z HSR vyvolávající ztrátu jednotlivých kopií nebo i celého HSR. Naproti tomu u linie SK-N-AS, která nemá *MYCN* amplifikován, jsme nalezli po inkubaci zvýšení počtu kopií *MYCN*. Po dlouhodobé kultivaci s cisplatinou, která indukuje chemorezistenci, se zvyšuje exprese MYCN přetrvávající i během kultivace bez cisplatiny. Vyšší expresi MYCN lze považovat za charakteristickou vlastnost chemorezistentních neuroblastomových buněk. Tyto výsledky jsou významné, protože v terapii neuroblastomu se uvažuje o využití onkogenu *MYCN* jako cíle cílené léčby např. zablokováním genu nebo utlumení jeho exprese RNA interferencí.

Výsledky prezentované v této práci rozšiřují poznání o chemorezistenci dětských nádorových onemocnění, která je jednou z nejvýznamnějších příčin selhání chemoterapie. Dále rozšiřují poznatky o mechanismech chemorezistence a genetických změnách spojených s jejím vznikem.

# 5 Literatura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