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Autoreferát disertační práce

Využití detekce cirkulujících a diseminovaných nádorových buněk v léčbě a diagnostice solidních tumorů

Nový prognostický a prediktivní biomarker v léčbě pacientů s maligním nádorovým onemocněním

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Obsah

Souhrn

Přestože nárůst incidence zhoubných novotvarů v České republice (ČR) během posledních let zpomalil, je v ČR průměrně diagnostikováno kolem 58 000 pacientů s maligním nádorovým onemocněním (mimo nemelanomové kožní novotvary) za rok. V souvislosti s ním cca 27 000 osob za rok také umírá (ÚZIS, 2022). Důležitým předpokladem úspěšné léčby nádorových onemocnění je nejen záchyt časných stadií, ale i nastavení optimální léčby individuálně pro každého pacienta.

Základní otázkou bylo, zda jsme CTC buňky schopni u různých typů nádorů zachytit, identifikovat a zjistit, jestli se jejich výskyt odráží ve stadiu onemocnění. Také nás zajímalo, jakou roli hrají CTC při sledování pacientů po operační léčbě a při nastavení terapie.

Do jednotlivých studií bylo za období 2013–2017 zařazeno 289 pacientů. Sběr dat probíhal retrospektivně. Přítomnost CTC byla prokázaná 75,3 % případech testovaných vzorků krve pacientů s různými druhy solidních nádorů. Dále byly CTC/DTC inkubovány jako krátkodobé i dlouhodobé buněčné kultury. Některé izolované CTC/DTC byly pěstovány *in vitro* déle než 6 měsíců. CTC pozitivita nekorelovala se stadiem onemocnění, velikostí nádoru nebo postižením lymfatických uzlin. Stejné procento pozitivity CTC bylo pozorováno u metastatických i non-metastatických (66,7 % vs. 66,7 %) pacientů s karcinomem pankreatu, u pacientů s kolorektálním karcinomem pak bylo pozorováno mírně vyšší procento pozitivity u metastatických než u non-metastatických (87,1 % vs. 80,6 %).

Překvapivým zjištěním, byl fakt, že CTC byly detekovány i v případech pacientek s preinvazivním stadiem karcinomu prsu (4,2 %).

Vyšetření CTC patří do skupiny testů tekuté biopsie a jednou z jeho hlavních výhod je relativně minimální invazivita (odběr krve). Použitá filtrační metoda založená na velikosti (MetaCell®) umožnila zavedení životaschopné kultury CTC *in vitro*, která byla nezbytná pro zjištění rozdílů mezi primárními nádorovými buňkami a CTC.

Z terapeutického hlediska bylo nejvýznamnějším zjištěním, že status HER2 a ER u CTC karcinomů prsu se může lišit od stavu těchto receptorů u primárního tumoru, tedy že charakter CTC se během sledovaného období mění a skutečně umožňuje sledování nádorové dynamiky.

Summary

The incidence of malignant neoplasms in the Czech Republic has been growing for a long time and every year more than 87,000 patients are diagnosed with cancer and on average 27,000 die due to cancer (ÚZIS, 2020). An important prerequisite for successful treatment of cancer is not only the detection of early stages, but also the setting of optimal treatment individually for each patient.

The basic question was whether we are able to capture, identify and find out whether CTC cells in different types of tumours are reflected in the stage of the disease. We were also interested in the role that CTCs play in monitoring patients after surgery and oncological treatment.

A total of 289 patients were included in the individual studies that took place during the years 2013 to 2017. Data collection took place retrospectively. Peripheral blood samples were collected from all patients, either on the day of surgery or during chemotherapy.

The presence of CTC was demonstrated in 75.3% of cases of blood samples tested from patients with various types of solid tumors. CTC/DTC were further incubated as both short-term and long-term cell cultures. Some isolated CTC/DTCs were grown in vitro for more than 6 months. CTC positivity did not correlate with disease stage, tumor size, or lymph node involvement. The same percentage of CTC positivity was observed in metastatic and non-metastatic patients with pancreatic cancer (66.7% vs. 66.7%). In patients with colorectal cancer a slightly higher percentage of positivity was observed in metastatic patients than in non-metastatic (87,1% vs. 80,6%).

A surprising finding was the fact that CTCs were detected in patients with pre-invasive breast cancer (4,2%).

CTC examination is a type of liquid biopsy test and one of its main advantages is relatively minimal invasiveness. The size-based filtration method used (MetaCell®) allows in vitro cell cultivation, which can be used to detect differences between primary tumor cells and CTC.

From a therapeutic point of view, the most significant finding was that the status of HER2 and ER may differ between CTC breast cancer and the primary tumor and that the characteristics of the CTC changed during the study period. These fact highlight the need for monitoring of tumor cell dynamics in clinical practise.

1 Úvod

Cirkulující nádorové buňky (CTC) a diseminované nádorové buňky (DTC) jsou zodpovědné za rozvoj metastazování nádorů. K úspěšné tvorbě nového metastatického nádorového ložiska je potřeba nejen CTC schopná tvořit metastázy, ale i vhodné orgánové mikroprostředí (stroma). Přes značnou morfologickou, fenotypovou a genetickou různorodost CTC je pouze malý počet z nich schopen přežít v krevním oběhu a vytvářet metastázy (Obrázek 1.).

Detekce a identifikace CTC schopných tvořit metastázy je nezbytná při vývoji terapeutických strategií proti diseminaci solidních nádorů. Rovněž je nezbytné zkoumání tumorigenního a metastatického potenciálu CTC schopných metastazovat v rámci *in vitro* a *in vivo* studií (Menyailo et al., 2020).
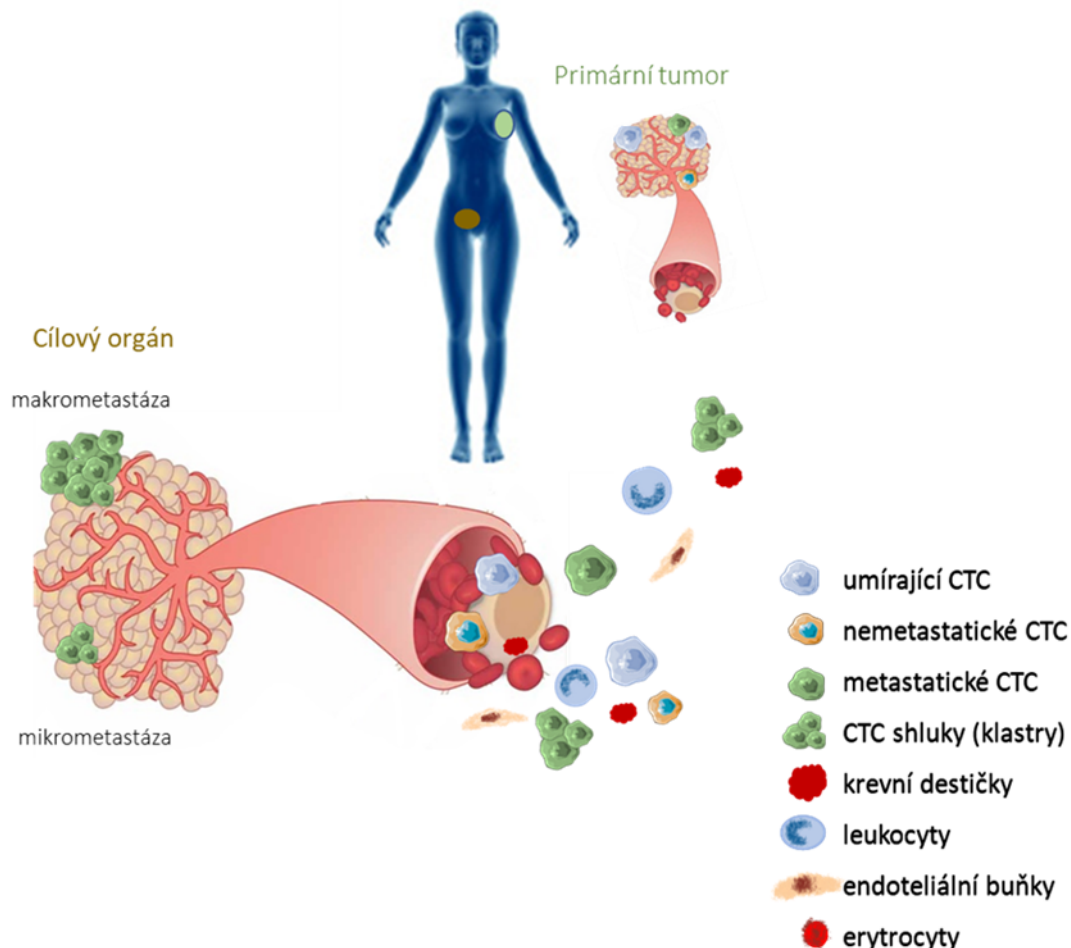
Obrázek 1**.** Metastatický proces nádorového onemocnění

2 Cíle práce a stanovené hypotézy

2.1 Cíle práce

Cílem práce bylo izolovat CTC z krve pacientů s různými druhy solidních tumorů a následně se pokusit o jejich kultivaci *in vitro* a cytomorfologickou charakterizaci.

Zlepšením a stanovením vhodných postupů při detekci CTC z periferní krve (tzv. liquid biopsy) bychom rádi přispěli k diagnostice a volbě vhodné terapie (včetně chirurgické), např. u pacientů, kterých není možné získat vzorky nádorové tkáně při chirurgickém zákroku. Obdobně monitorace efektu chirurgické léčby pomocí CTC a záchyt časné rekurence nádorového onemocnění (kdy ještě nejsou přítomny metastázy) představují další z možných způsobů využití těchto nádorových elementů.

Ze skupiny pacientů se solidními tumory jsme se v rámci práce zaměřili na pacienty s nádory gastrointestinálního traktu a prsu. U pacientek s karcinomem prsu bylo cílem pokusit se o molekulární charakterizaci cirkulujících nádorových buněk v průběhu léčby.

Záměrem rovněž bylo zjistit, zda je pomocí identifikace CTC a specifikace vztahu výskytu CTC a stadia nemoci možné rozdělit pacienty do prognostických skupin.

2.2 Stanovené hypotézy

Hypotéza I: Vyskytují se CTC u různých druhů solidních tumorů?

Základem léčby všech solidních nádorů je jejich chirurgické odstranění. Otázkou, kterou si klademe, je, zda výskyt CTC souvisí s určitým histologickým typem tumoru.

Hypotéza II: Odráží se ve výskytu CTC stadium nádorového onemocnění?

V klinické praxi slouží k jednoduchému popisu rozsahu nádoru a určení stadia onemocnění TNM systém (klinická klasifikace cTNM a patologická klasifikace pTNM). Staging nádorového onemocnění je jedním z kritérií, podle kterého se lékař rozhoduje o načasování a typu vhodné terapie: lokoregionální (chirurgický výkon, radioterapie) či systémové. Restaging v průběhu léčby či po jejím ukončení monitoruje odpověď nádorového onemocnění na léčbu. S ohledem na skutečnost, že přítomnost CTC v krvi doprovází šíření maligního onemocnění hematogenní cestou, nás zajímá, zda výskyt CTC koreluje se stadiem nádorového onemocnění.

Hypotéza III: Lze použít CTC k molekulární charakterizaci nádorového onemocnění?

Profily subpopulací CTC představují prostředek identifikace individuálních změn v kancerogenezi a citlivosti na léčbu (tzv. real-time nádorová biopsie) a díky tomu vhodný biomarker pro monitoraci nádorového onemocnění v průběhu léčby.

Hypotéza IV: Je možné vizualizovat CTC/DTC ve viabilním stavu?

Stanovení viability nádorových buněk je založeno na detekci životně důležitých funkcí pomocí selektivního značení fluorescenčními barvami (např. měřením intracelulární esterázové aktivity či sledováním membránové integrity). Ke sledování značených buněk je tradičně využíván fluorescenční mikroskop, který poskytuje náhled na jednotlivé buňky a umožňuje analýzu jejich vlastností.

3 Materiál a metodika

V rámci testování CTC byla všem pacientům odebrána periferní krev (2 x 8 ml, EDTA), a to buď v den operace, nebo v průběhu chemoterapie. Pro obohacení CTC byl použit separační protokol založený na velikosti buněk (MetaCell®). Po jednoduché filtraci krve přes polykarbonátovou membránu s póry byly zachycené buňky (větší než 8 μm) inkubovány *in vitro* po krátkou dobu (3–5 dnů) či dlouhodobě (déle než 5 dní). Po krátkodobé inkubaci byly buňky barveny histochemickým barvením fixovaných buněk (např. barvení May-Grünwald-Giemsa), imunohistochemickým barvením pomocí specifických protilátek k identifikaci buněčných organel (př. anti-cytokeratin 18-FITC) či jádra (DAPI) a vitálními fluorescenčními barvivy (NucBlue™, Celltracker™). Přítomnost CTC byla vyhodnocena na základě cytomorfologické charakterizace podle definovaných histopatologických kritérií. Část vzorků (43) byla využita k identifikaci CTC pomocí molekulární analýzy (qPCR) (Jakabova et al., 2017).

Vizualizace a cytomorfologická charakterizace CTC

Prvním krokem v analýze separovaných buněk bylo popsání morfologie buněk na základě cytomorfologických parametrů pomocí světelného či fluorescenčního mikroskopu ve dvou krocích:

1. skríning při 20násobném zvětšení pro lokalizaci buněk,
2. při 40–60násobném zvětšení pro detailní cytomorfologickou analýzu.

Izolované buňky nebo shluky buněk byly vybrány, digitalizovány a zhodnoceny zkušeným výzkumníkem, cytologem či patologem. CTC byly definovány jako buňky splňující následující histopatologická kritéria pro CTC:

1. viditelná cytoplazma – velké množství cytoplazmy (velikost buňky > 15 μm),
2. nepravidelnost okrajů jaderné membrány,
3. velikost jádra stejná nebo větší než 10 μm,
4. prominentní nukleoly (jadérka),
5. vysoký poměr mezi velikostí jádra a cytoplazmy – N/C poměr,
6. přítomnost 2D/3D struktur proliferujících buněk – proliferace, plasticita.

Molekulární charakterizace nádorových buněk – analýza genové exprese

Pro stanovení molekulárního profilu CTC u karcinomu prsu byl použit multimarker panel genů asociovaných s tumory (tzv. tumor asociované geny) a s terapeutickým potenciálem: Actin, CD45, CD68, EPCAM, MUC1, KRT18, KRT19, ESR, PGR, mamaglobin, HER2, CD24, CD44. Kromě toho byly testovány geny spojené s chemoresistencí (MRP1-10, MDR1, ERCC1). Geny byly do panelu vybrány na základě standardních postupů diferenciální diagnostiky v cytopatologii při určování typů tumorů s neznámým původem.

Pro sledování přítomnosti a frekvence mutací genu ESR*/Her* bylo potřeba analyzovat DNA z primárního nádoru (DNeasy, Qiagen, Německo). Dále byly stanovovány exprese genů na úrovni messenger RNA (mRNA) v nádorových buňkách v periferní krvi. U vzorků pozitivních na CTC nebo DTC byla RNA přepsána reverzní transkripcí do komplementární DNA (cDNA). Stanovení expresí genů proběhlo pomocí qPCR (quantitative polymerase chain reaction).

Statistická analýza

Statistická analýza byla provedena pomocí klinickopatologických informací transformovaných na proměnné 0 a 1, pokud byly použitelné pro testované vlastnosti. Chi-čtvercový (Chi Squared) test byl použit k určení, zda existuje významný vztah mezi dvěma nominálními (kategorickými) proměnnými (např. přítomnost metastázy a CTC-pozitivita). Statisticky významná incidence rozdílů mezi dvěma porovnanými skupinami a přítomností CTC byla stanovena na $p < 0,05$.

4 Výsledky

4.1 Izolace cirkulujících/diseminovaných nádorových buněk z krve u pacientů s různými druhy solidních tumorů

Přítomnost CTC/DTC byla prokázaná v 75,3 % případech z celkového počtu 298 testovaných vzorků krve pacientů, kteří podstoupili chirurgický zákrok či systémovou léčbu pro malignitu. K izolaci CTC/DTC jsme použili separační metodu založenou na velikosti (MetaCell$^{\text{TM}}$) a úspěšně jsme tímto způsobem detekovali CTC/DTC u pacientů s různými druhy zhoubných solidních nádorů – s karcinomem prsu (přítomnost v 72,1 % ze 165 pacientek), karcinomem pankreatu (přítomnost v 66,7 % z 24 pacientů), karcinomem tlustého střeva (přítomnost v 88,9 % ze 45 pacientů) a karcinomem rektosigmoidea (přítomnost v 77,4 % z 53 pacientů) (Bobek et al., 2014; Eliášová et al., 2017; Jakabová et al., 2017).

U pacienta s inoperabilním NET tenkého střeva (tkáňová histologická verifikace metastázy peritonea – Obrázek 2.) jsme izolovali DTC z peritoneální laváže získané při operaci (Obrázek 3.).
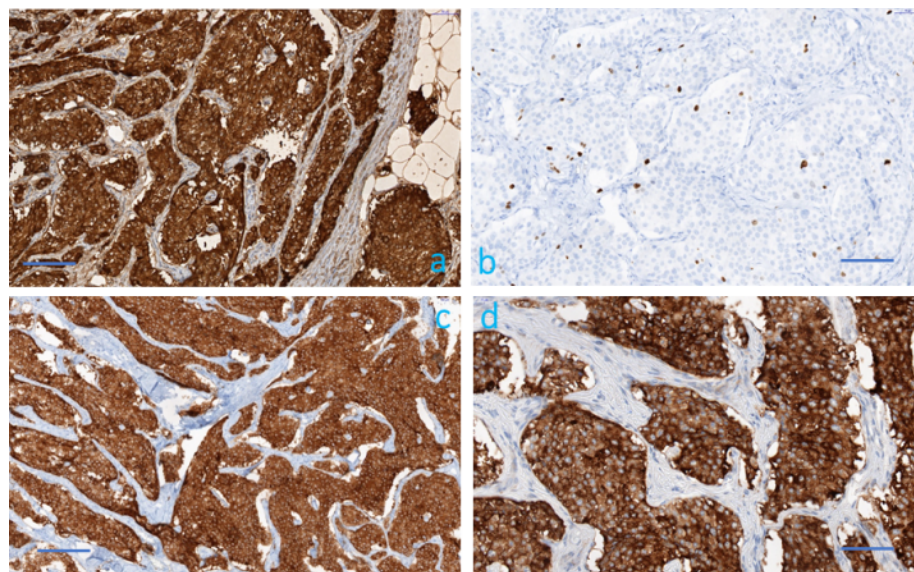
Obrázek 2. Imunohistochemické vyšetření tkáně primárního nádoru pacienta s inoperabilním neuroendokrinním tumorem, a) chromogranin, b) proliferační aktivita (Ki67 20 %), c) serotonin, d) CDX 2-marker specifický pro střevní epitel
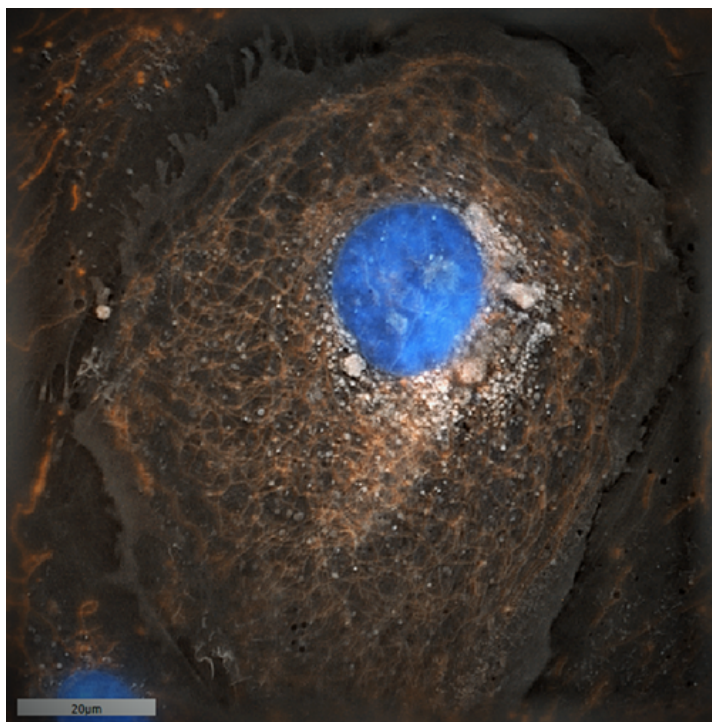
Obrázek 3. Diseminovaná nádorová buňka izolovaná a kultivovaná in vitro z peritoneální laváže pacienta s inoperabilním neuroendokrinním tumorem

4.2 Výskyt cirkulujících nádorových buněk a rozdělení pacientů do prognostických skupin

Do studií byli zařazeni pacienti s diagnostikovanou malignitou v různém stadiu onemocnění, od kterého se odvíjela i jejich prognóza. Prognostické faktory jako velikost tumoru, přítomnost metastáz v regionálních uzlinách či ve vzdálených orgánech a histologický typ nádoru či jeho grade jsme se snažili dát do souvislosti se shromážděnými údaji o CTC pozitivitě. Naším překvapivým zjištěním byl fakt, že CTC pozitivita nekorelovala se stadiem onemocnění a že jsme u pacientek s karcinomem prsu detekovali CTC i v preinvazivním stadiu 0 (karcinom *in situ*) (Tab. 1, Bobek et al., 2014; Tab. 1, Eliášová et al., 2017; Tab. 2, Jakabová et al., 2017).

Stejné procento pozitivity CTC jsme pozorovali u metastatických i non-metastatických pacientů s karcinomem pankreatu (66,7 % vs. 66,7 %) (Tab. 1, Bobek et al., 2014). U pacientů s kolorektálním karcinomem bylo procento pozitivity u metastatických o něco vyšší než u non-metastatických nádorů (87,1 % vs. 80,6 %), ale ani hodnocení výskytu vzdálených metastáz ani metastatické postižení uzlin (u N0 pacientů pozitivita CTC v 82,7 % vs. 85 % u N1 pacientů) neprokázalo žádný statisticky významný rozdíl ve vztahu s výskytem CTC v krvi (p $\geq$ 0,05) (Tab. 1, Eliášová et al., 2017).

Obdobně u kohorty pacientek s karcinomem prsu nebyl zjištěn žádný významný rozdíl mezi testovanými podskupinami s rozdílnou prognózou. Nejvyšší výskyt CTC byl pozorován ve skupině pacientek před operací (86,6 %) nebo před zahájením neoadjuvantní léčby (82,3 %).

4.3   Molekulární charakterizace cirkulujících nádorových buněk v průběhu léčby pacientek s karcinomem prsu

Část vyšetřovaných pacientek s karcinomem prsu (n = 20) jsme pravidelně v průběhu onemocnění a léčby sledovali a mimo přítomnost CTC jsme zkoumali také molekulární charakteristiku CTC se zvláštním zaměřením na stav receptorů HER2 a ER. Analýza genové exprese odhalila (celkem 43 qPCR vyšetření), že status HER2 a ER se u CTC může lišit od jejich statusu u primárního tumoru.

Nejčastější změny byly pozorovány ve skupině pacientek s triple negativním karcinomem prsu (ER-/PR-/HER2-; n = 12), u kterých bylo hodnoceno 27 vzorků. U 4 z 12 pacientek jsme zachytili změnu HER2, a to z HER2 – stavu detekovaného z tkáně primárního tumoru na HER2+ detekovaný z CTC. Změna byla relevantní pro 33, 3 %, což je již významným číslem. Podobně se u dalších 50 % pacientek status HER2 změnil z HER2+ u primárního tumoru na HER2-CTC (3 z 6 pacientek). Stav ER v CTC se změnil pouze v jednom směru, a to z ER+ na ER- (3 z 3 pacientek). Toto zjištění potvrdilo naši hypotézu, že CTC lze využít k molekulární charakterizaci nádorového onemocnění (Jakabová et al., 2017).

4.4   Kultivace *in vitro* a cytomorfologická charakterizace CTC/DTC diseminovaných nádorových buněk

Po izolaci CTC/DTC jsme za předem definovaných podmínek úspěšně založili životaschopné buněčné kultury *in vitro*, které byly dále inkubovány jako krátkodobé (do 5 dní) nebo dlouhodobé (déle než 5 dní) kultury. Některé izolované DTC byly pěstovány a proliferovaly *in vitro* i déle než 6 měsíců (Bobek et al., 2014; Eliášová et al., 2017; Jakabová et al., 2017).

Ke stanovení viability nádorových buněk jsme použili detekci životně důležitých funkcí buněk pomocí selektivního značení fluorescenčními barvivy, např. sledování membránové integrity a buněčného dělení prostřednictvím sledovaní jaderné morfologie (NucBlue®) či pohybu buněk (Celltracker®). Ke sledování značených buněk jsme použili fluorescenční mikroskop, který nám poskytl náhled na jednotlivé buňky a umožnil analýzu jejich

cytomorfologie a chování (Obrázek 1., Eliášová et al., 2017; Obrázek 1., Jakabová et al., 2017). Životaschopné CTC/DTC jsme úspěšně zachytili a zobrazili u pacientů se všemi typy nádorů, které jsme vyšetřovali. V řadě případů jsme byli schopni zachytit i proliferaci nádorových buněk a jejich přesun přes póry separační membrány (Obrázek 1., Bobek et al., 2014).

5 Diskuse

5.1 CTC u různých druhů solidních tumorů – diagnostický biomarker

Detekce a charakterizace CTC může přispět ke včasné diagnostice, pochopení nádorového onemocnění a zefektivnění terapie. Klíčovým krokem je použití dostatečně citlivé metody izolace a detekce CTC. Vysoká heterogenita CTC musí být zohledněna před výběrem izolačních a obohacujících metod za účelem minimalizace chybných postupů v detekci CTC hned v počátku.

Separační filtrační metody nezávislé na protilátkách se ukazují účinnější při identifikaci CTC, neboť izolační metody CTC založené pouze na detekci epiteliálních markerů (např. EpCAM, cytokeratiny) nezachytí všechny CTC v důsledku jejich změn, které podstupují v rámci EMT procesu (Cohen et al., 2008; Cohen et al., 2009). Byly uveřejněny relativně nízké hodnoty záchytu CTC pomocí metod izolace vycházejících ze záchytu exprese EpCAM v časných fázích onemocnění (např. CellSearch®, Adnagen®). Ukazuje se, že izolační protokoly EpCAM nemusí být pro CTC detekci dostatečně senzitivní (Farace et al., 2011; Hofman et al., 2011; Sandri et al., 2010).

Mnoho úsilí bylo v posledním desetiletí věnováno vývoji nových „non-EpCAM" detekčních metod. Jejich další vývoj je konfrontován s dalšími výzvami, jako jsou zlepšení míry čistoty a obohacení získaného vzorku, zajištění životaschopnosti CTC a udržení intaktního buněčného povrchu izolovaných CTC (Gabriel et al., 2016).

Wang et al. (2016) přednáší k další diskusi problematiku kultivování izolovaných CTC pro klinické účely pouze z periferní krve. S ohledem na strukturu cirkulačního systému poukazuje na skutečnost, že ne všechny populace CTC jsou rovnoměrně rozloženy v krvi a použití samotné periferní krve k detekci CTC nemůže představovat celou genetickou variabilitu cirkulující nádorové buněčné populace (Wang et al., 2016).

Námi prezentovaný soubor studií je zaměřen na úspěšnou izolaci CTC od pacientů se zhoubnými nádory gastrointestinálního traktu (slinivky, tlustého a tenkého střeva) a prsu pomocí jednoduché separační metody založené na velikosti (MetaCell®) (Cegan et al., 2014, Kolostová et al., 2016).

Nízká specificita je bohužel největší nevýhodou námi použité separační metody založené na velikosti. Oproti tomu výhodou je její vysoká citlivost a šetrná izolace životaschopných

buněk s možnou následnou kultivací. Izolace celých buněk poskytuje další možnost molekulární charakterizace k jejich specifikaci. Jak kultivace s vyhodnocením CTC cytomorfologie dle histopatologických kritérií, tak molekulární charakterizace pak umožňují přesnou identifikaci CTC/DTC a jejich využití jako diagnostického biomarkeru (Bobek et al., 2014; Eliášová et al., 2017; Jakabová et al., 2017).

V korelaci s našimi daty byly obdobnou metodikou detekovány a identifikovány CTC u dalších typů solidních nádorů, a to nádorů jícnu (Bobek et al., 2014), žaludku (Kolostová et al., 2016), močového měchýře (Cegan et al., 2014) či ledviny (Klézl et al., 2020).

5.2 Cirkulující nádorové buňky a stadia nádorového onemocnění – prognostický biomarker

Ve snaze zhodnotit prognostický význam CTC pozitivity byla publikována celá řada studií (Eliášová et al., 2013) a meta-analýz o možném využití CTC jako prognostického markeru. Příkladem uvádíme metaanalýzu Rahbari et al. (2012) u pacientů s kolorektálním karcinomem, kde bylo zahrnuto 36 studií (n = 3 094 pacientů). Tyto studie kombinovaly různá místa odběru krve z krevního oběhu (periferní krev, mesenterická krev), přičemž je pojil vztah pozitivity CTC/DTC a kratší doby přežití bez návratu nemoci. Také bylo prokázáno, že detekce CTC z periferní krve byla statisticky významným prognostickým faktorem (Rahbari et al., 2012).

U pacientů s detekovatelnými CTC bylo zjištěno významně horší celkové přežití a kratší doba do relapsu nemoci (Iinuma et al., 2011). Z výše uvedených publikací je zřejmé, že detekce CTC je silným prognostickým parametrem u pacientů s pokročilým nádorovým onemocněním a dynamika změn CTC může napomoci ke stratifikaci těchto pacientů. Překvapivým zjištěním byl fakt, že CTC pozitivita nekorelovala se stadiem nádorového onemocnění (Tab. 1, Bobek et al., 2014; Tab. 1, Eliášová et al., 2017; Tab. 2, Jakabová et al., 2017).

Dříve se předpokládalo, že pouze u invazivních nádorů může docházet k uvolňování izolovaných nádorových buněk do krevního oběhu a lymfatických cest. Podle našich výsledků a také ve shodě se zahraničními studiemi se ukazuje možná diseminace nádorových buněk dokonce u karcinomů *in situ* (Jakabová et al., 2017; Banys, 2012; Sanger, 2011). To by znamenalo, že nádorové buňky mohou diseminovat již z preinvazivních mammárních lézí, ze skrytých invazivních nádorů nebo dokonce se může jednat o časný krok mikroinvaze z preinvazivních lézí. Proto se v současné době výzkumné aktivity zaměřují na detekci

a charakterizaci CTC u časných stadií nádorových onemocnění (Thery et al., 2019). V naší studii u kohorty pacientek s časným karcinomem prsu bylo více než 68 % případů CTC pozitivních (Jakabová et al., 2017). Tento výsledek poskytuje důkaz, že CTC migrují a šíří se i z morfologicky velmi raných lézí. Hosseini et al. (2016) prokázal, že metastatické šíření se často vyskytuje brzy během tvorby nádoru (Hosseini et al., 2016). Cirkulující nádorové buňky detekované u pacientek před projevem metastázy rakoviny prsu obsahují méně genetických abnormalit než primární nádory a ukazují, že k šíření dochází i během raných fází růstu nádoru (Schardt et al., 2005; Schmidt-Kittler et al., 2003; Husemann et al., 2008; Sanger et al., 2011). Přestože postavení CTC jako nezávislého prognostického biomarkeru je potvrzeno, k možnému použití detekce CTC jako optimálního nástroje k záchytu časných stadií či monitorování minimální reziduální choroby je potřeba dalších klinicky podložených výsledků.

5.3 Molekulární analýza a identifikace cirkulujících nádorových buněk – prediktivní biomarker

Výzkum na poli CTC pokročil z kvantifikace izolovaných CTC buněk k jejich molekulární charakterizaci, což by mělo umožnit monitoraci vývoje fenotypu nemoci v čase a poskytnout potenciální prediktivní marker odpovědi na léčbu. Molekulární charakterizace vychází z konceptu EMT, kdy buňka primárního tumoru ztrácí vlastnosti epiteliální buňky a získává některé vlastnosti mesenchymální buňky. Současně se mění genetický profil a signální dráhy, které jsou zodpovědné za inhibici apoptózy, proliferaci a metastatické vlastnosti CTC (Liberko et al., 2013).

Rozhodnutí o léčbě karcinomu prsu jsou založena na vlastnostech primárního nádoru, aniž by zohledňovala charakter metastáz či minimální reziduální chorobu. Nakolik se však vyvíjejí entita a genetická heterogenita, bylo zjišťováno porovnáním tkáně primárního nádoru s metastázami a analýzou různých oblastí stejného nádoru (Siu et al., 2013). Bylo předpokládáno, že úspěch personalizovaných léčebných postupů velmi závisí na schopnosti zachytit a monitorovat heterogenitu nádoru v průběhu času a následně modulovat terapeutické postupy (Gagan et al., 2015). Je možné identifikovat v CTC citlivé geny, které by mohly být použity pro vývoj a optimalizaci genové terapie a imunoterapie u jednotlivých pacientů (Cen et al., 2012).

Právě CTC mohou pomoci identifikovat rozdíly mezi primárním nádorem a metastázami na úrovni fenotypu a genotypu. Klonální heterogenita primárního nádoru by mohla vysvětlit rozdíly mezi populacemi DTC a vývojem jejich nových genotypů a fenotypů (Han et al., 2014). Proto identifikace rozdílů na úrovni genové exprese mezi primárními nádorovými buňkami a CTC by měla být základem pro zlepšení terapeutických přístupů.

Signifikantním zjištěním vycházejícím z našeho výzkumu bylo, že analýza genové exprese odhalila (celkem 43 qPCR vyšetření) odlišný stav receptorů HER2 a ER u CTC od jejich stavu u primárního tumoru (Jakabová et al., 2017). Molekulární profil CTC informuje o aktuálním stavu receptorů na CTC, což umožňuje monitorování onemocnění (real-time biopsy) a může vést k individuálnímu cílení onkologické léčby.

5.4 Vizualizace viabilních cirkulujících a diseminovaných nádorových buněk – kultivace *in vitro*

Založení životaschopné buněčné kultury *in vitro*, a to jak krátkodobé, tak dlouhodobé (Bobek et al., 2014; Eliášová et al., 2017; Jakabová et al., 2017), bylo základem pro další analýzu cytomorfologie a chování CTC/DTC u různých typů solidních nádorů (Bobek et al., 2014; Eliášová et al., 2017; Jakabová et al., 2017). Viabilita nádorových buněk byla hodnocena pomocí značení fluorescenčními barvivy (NucBlue®, Celltracker®).

Studie *in vitro* mohou být užitečné pro pochopení proliferativního a apoptotického potenciálu CTC, také pro jejich migrační a invazní schopnosti aj. Nicméně je potřeba přihlédnout k faktu, že v buněčných kulturách při kultivaci *in vitro* je uměle vytvořené prostředí, které neodráží skutečný obraz růstu nádoru v živém organismu (odlišná dostupnost kyslíku, živin, metabolitů a signalizačních molekul). Nedochází tedy k plnohodnotné interakci nejen mezi nádorovými, imunitními a stromálními buňkami a extracelulární matrix, ale i mezi nádorovými buňkami navzájem. To vše hraje důležitou roli v buněčné diferenciaci a proliferaci (Thoma et al., 2014). Také přilnutí buňky k plastovému povrchu může způsobit změnu morfologie nádorové buňky, což může ovlivnit organizaci intracelulárních struktur buňky s narušením jejích funkcí (Delarue et al., 2014).

Zavedení, kultivace *in vitro* a následný výzkum 3D buněčných kultur, jako jsou clustery, nádorové sféroidy či organoidy (trojrozměrné buněčné modely skládající se pouze z nádorových buněk nebo jejich kombinace s jinými typy buněk) jsou další možnou cestou preklinického výzkumu CTC/DTC (Guo et al., 2016; Gabriel et al., 2016).

6 Závěr

Předkládaná disertační práce shrnuje dílčí výzkumné práce, jejichž cílem bylo izolovat CTC z krve pacientů s různými druhy solidních tumorů a následně se pokusit o jejich kultivaci *in vitro* a cytomorfologickou charakterizaci. Do studií bylo za období 2013–2017 zařazeno celkem 289 pacientů.

Práce zodpověděla následující hypotézy:

Hypotéza I: Vyskytují se CTC u různých druhů solidních tumorů?

Studie prokázaly výskyt CTC u různých druhů solidních tumorů. Použili jsme jednoduchou separační metodu s nízkou specificitou a vysokou senzitivitou záchytu CTC. Touto metodou byly CTC detekovány a izolovány u různých histopatologických druhů nádorů.

Hypotéza II: Odráží se ve výskytu CTC stadium nádorového onemocnění?

U pacientů s detekovatelnými CTC bylo zjištěno významně horší celkové přežití a kratší doba do relapsu onemocnění. Detekce CTC je silným prognostickým parametrem u pacientů s pokročilým nádorovým onemocněním a dynamika změn CTC může napomoci ke stratifikaci těchto pacientů. Překvapivým zjištěním výzkumu byl fakt, že CTC pozitivita nekorelovala se stadiem nádorového onemocnění.

Hypotéza III: Lze využít CTC k molekulární charakterizaci nádorového onemocnění?

Ano, podařilo se identifikovat v CTC geny, které by mohly být použity pro vývoj a optimalizaci genové terapie a imunoterapie u jednotlivých pacientů. Tímto CTC mohou pomoci identifikovat rozdíly mezi primárním nádorem a metastázami na úrovni fenotypu a genotypu. Molekulární profil CTC informuje o aktuálním stavu receptorů na CTC, což umožňuje monitorování onemocnění (real-time biopsy) a může vést k individuálnímu cílení onkologické léčby.

Hypotéza IV: Je možné vizualizovat CTC/DTC ve viabilním stavu?

Ano, podařilo se založení životaschopné buněčné kultury *in vitro*, a to jak krátkodobé, tak dlouhodobé, které bylo základem pro další analýzu cytomorfologie a chování CTC/DTC u různých typů solidních nádorů.

7 Použitá literatura

BANYS M., GRUBER I., KRAWCZYK N. et al.; Hematogenous and lymphatic tumor cell dissemination may be detected in patients diagnosed with ductal carcinoma in situ of the breast. Breast Cancer Res. Treat., 2012; 131, 801-808.80. ISSN: 0167-6806 (Print). ISSN: 1573-7217 (Electronic). DOI:10.1007/s10549-011-1478-2.

BOBEK V., GÜRLICH R., ELIASOVA P., KOLOSTOVA K.; Circulating tumor cells in pancreatic cancer patients: enrichment and cultivation. World J Gastroenterol, 2014 Dec 7;20(45):17163-17170. ISSN:1007-9327 (Print) ISSN: 2219-2840 (Electronic). DOI: 10.3748/wjg. v20. i45.17163.

CEN P., NI X., YANG J. et al.; Circulating tumor cells in the diagnosis and management of pancreatic cancer. Biochim Biophys Acta, 2012; 1826: 350-356. ISSN: 0006-3002 (Print). ISSN: 1878-2434 (Electronic). DOI:10.1016/j.bbcan.2012.05.007.

COHEN S.J., PUNT C.J.A., IANNOTTI N. et al.; Relationship of cir-culating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastat-ic colorectal cancer. J Clin Oncol. ,2008; 26(19): 3213-3221. ISSN:0732-183X (Print). ISSN:1527-7755 (Electronic).DOI:10.1200/JCO.2007.15.8923.

COHEN S.J., PUNT C.J.A., IANNOTTI N. et al.; Prognostic significance of circulating tumor cells in patients with metastatic colorec-tal cancer. Ann Oncol., 2009; 20(7): 1223–9. ISSN: 0923-7534 (Print). ISSN: 1569-8041 (Electronic). DOI:10.1093/annonc/mdn786.

ELIASOVA P., KOLOSTOVA K., KOBIERZYCKI C., BOBEK V.; Clinical studies monitoring circulating and disseminated tumor cells in gastrointestinal cancers. Folia Histochem Cytobiol.,2013;51(4),265-277. ISSN: 0239-8508 (Print). ISSN: 1897-5631 (Electronic). DOI: 10.5603/FHC.2013.0037.

ELIASOVA P., PINKAS M., KOLOSTOVA K., GURLICH R., BOBEK V.; Circulating tumor cells in different stages of colorectal cancer. Folia Histochem Cytobiol., 2017;55(1):1-5. ISSN: 0239-8508 (Print). ISSN: 1897-5631 (Electronic). DOI: 10.5603/FHC.a2017.0005.

FARACE F., MASSARD C., VIMOND N. et al.; A direct com-parison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. Br J Cancer, 2011; 105: 847-853. ISSN:0007-0920 (Print).ISSN:1532-1827 (Electronic). DOI: 10.1038/bjc.2011.294].

GABRIEL M.T., CALLEJA L.R., CHALOPIN A. et al.; Circulating Tumor Cells: A Review of Non-EpCAM-Based Approaches for Cell Enrichment and Isolation.Clin.Chem.,2016; 62,571-581. ISSN: 0009-9147 (Print). ISSN: 1530-8561 (Electronic). DOI: 10.1373/clinchem.2015.249706.

GAGAN J., VAN ALLEN E.M.; Next-generation sequencing to guide cancer therapy. Genome Med., 2015 Jul; 29;7(1):80. ISSN: 1756-994X (Electronic). DOI: 10.1186/s13073-015-0203-x.

GUO T.; WANG, C. S.; WANG, W. et al.; Culture of Circulating Tumor Cells—Holy Grail and Big Challenge. Int. J. Cancer Clin. Res., 2016; 3, 065. ISSN: 2378-3419 (Electronic). DOI: 10.23937/2378-3419/3/4/1065.

HAN L., CHEN W., ZHAO Q.; Prognostic value of circulating tu-mor cells in patients with pancreatic cancer: a meta-analysis. Tumour Biol., 2014; 35: 2473-2480. ISSN: 1010-4283 (Print). ISSN: 1423-0380 (Electronic). DOI: 10.1007/s13277-013-1327-5.

HOFMAN V., ILIE MI, LONG E., SELVA E. et al.; Detection of circulating tumor cells as a prognostic factor in patients un-dergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay™ and the isolation by size of epithelial tumor cell method. Int J Cancer, 2011; 129: 1651-1660. ISSN: 0020-7136 (Print). ISSN: 1097-0215 (Electronic). DOI:10.1002/ijc.25819]

HÜSEMANN Y. et al.; Systemic spread is an early step in breast cancer. Cancer Cell,2008; 13:58-68. ISSN: 1535-6108 (Print). ISSN: 1878-3686 (Electronic). DOI: 10.1016/j.ccr.2007.12.003

IINUMA H., WATANABE T., MIMORI K. et al.; Clinical significance of circulating tumor cells, including cancer stem-like cells, in peripheral blood for recurrence and prognosis in patients with Dukes' stage B and C colorectal cancer. J. Clin Oncol., 2011; 29, 1547-1555. ISSN: 0732-183X (Print). ISSN: 1527-7755 (Electronic). DOI: 10.1200/JCO.2010.30.5151.

JAKABOVA A., BIELCIKOVA Z., POSPISILOVA E., MATKOWSKI R., SZYNGLAREWICZ B., STASZEK-SZEWCZYK U., ZEMANOVA M., PETRUZELKA L., ELIASOVA P., KOLOSTOVA K., BOBEK V.; Molecular characterization and heterogeneity of circulating tumor cells in breast cancer. Breast Cancer Res Treat. 2017 Dec;166(3):695-700. ISSN: 0167-6806 (Print). ISSN: 1573-7217 (Electronic). DOI: 10.1007/s10549-017-4452-9.

KLEZL P., POSPISILOVA E., KOLOSTOVA K. et al.; Detection of Circulating Tumor Cells in Renal Cell Carcinoma: Disease Stage Correlation and Molecular Characterization. J. Clin. Med., 2020; 7;9(5):1372. ISSN: 2077-0383 (Electronic). DOI:10.3390/jcm9051372

KOLOSTOVA K., PINKAS M., JAKABOVA A., POSPISILOVA E., SVOBODOVA P., SPICKA J., CEGAN M., MATKOWSKI R., BOBEK V.; Molecular characterization of circulating tumor cells in ovarian cancer. Am J Cancer Res., 2016; 6(5):973-980. ISSN: 2156-6976 (Electronic).

LIBERKO M., KOLOSTOVA K., BOBEK V.; Essentials of circulating tumor cells for clinical research and practice. Crit Rev On-col Hematol., 2013; 88(2): 338–356. ISSN: 1040-8428 (Print). ISSN: 1879-0461 (Electronic). DOI: 10.1016/j.critrev-onc.2013.05.002.

MENYAILO M.,TRETYAKOVA M. ,DENISOV; Heterogeneity of Circulating Tumor Cells in Breast Cancer: Identifying Metastatic Seeds.*Int. J. Mol. Sci.*, 2020; *21*(5), 1696. ISSN: 1661-6596 (Print). ISSN: 1422-0067 (Electronic). DOI: 10.3390/ijms21051696.

RAHBARI NN, AIGNER M, THORLUND K et al. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. *Gastroenterology*. 2010;138:1714–1726. ISSN 0016-5085. doi: 10.1053/j.gastro.2010.01.008.

SANDRI M.T., ZORZINO L., CASSATELLA M.C. et al.; Changes in circulating tumor cell detection in patients with localized breast cancer before and after surgery. Ann Surg. Oncol., 2010; 17: 1539-1545. ISSN: 1068-9265 (Print). ISSN: 1534-4681 (Electronic). DOI: 10.1245/s10434-010-0918-2

SÄNGER N., EFFENBERGER K. E., RIETHDORF S., VAN HAASTEREN V., GAUWERKY J., WIEGRATZ I., STREBHARDT K., KAUFMANN M., & Pantel, K.; Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. Int. J.Cancer ,2011; 15;129(10):2522-6. ISSN: 0020-7136 (Print). ISSN: 1097-0215 (Electronic), DOI: 10.1002/ijc.25895.

THERY L., MEDDIS A., CABEL L. et al.; Circulating Tumor Cells in Early Breast Cancer; JNCI Cancer Spectrum, 2019; 27,3(2), pkz026. ISSN: 2515-5091 (Electronic). DOI: 10.1093/jncics/pkz026.

SCHARDT J.A. et al.; Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. Cancer Cell, 2005; 8:227-239. DOI: 10.1016/j.ccr.2005.08.003

SCHMIDT-KITTLER O. et al.; From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer pro-gression. Proc Natl Acad Sci USA, 2003; 100:7737-7742. ISSN: 0027-8424 (Print). ISSN: 1091-6490 (Electronic). DOI: 10.1073/pnas.1331931100.

THOMA C.R., ZIMMERMANN M., AGARKOVA I., KELM, J.M., KREK W.; 3D cell culture systems modeling tumor growth determinants in cancer target discovery. Adv. Drug Deliv. Rev, 2014; 69-70:29-41. ISSN: 0169-409X (Print). ISSN: 1872-8294 (Electronic). DOI: 10.1016/j.addr.2014.03.001.

WANG R., CHU G. C. Y., MRDENOVIC S. et al.; Cultured circulating tumor cells and their derived xenografts for personalized onkology. Asian J.Urol.,2016; 3,240-253. ISSN: 2214-3882 (Print). ISSN: 2214-3890 (Electronic). DOI:10.1016/j.ajur.2016.08.005.

8 Publikace autora

8.1 Publikace *in extenso*, které jsou podkladem disertace s IF

ELIASOVA P., PINKAS M., KOLOSTOVA K., GURLICH R., BOBEK V.; Circulating tumor cells in different stages of colorectal cancer. *Folia Histochem Cytobiol.,* 2017;55(1):1-5. ISSN: 0239-8508 (Print), ISSN: 1897-5631 (Electronic). DOI: 10.5603/FHC.a2017.0005. **IF:** 1.389/2016.

ORIGINAL PAPER

V M
VIA MEDICA

Circulating tumor cells in different stages of colorectal cancer

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Abstract

Introduction. Liquid biopsies are noninvasive tests using blood or body fluids to detect circulating tumor cells
(CTCs) or the products of tumor cells, such as fragments of nucleic acids or proteins that are shed into biolo-
gical fluids from primary tumor or its metastates. The analysis of published clinical studies provides coherent
evidence that the presence of CTCs detected in peripheral blood is a strong prognostic factor in patients with
colorectal carcinoma (CRC). The aim of the study was to implement size-based separation protocol of CTCs
in CRC patients.

Material and methods. Patients diagnosed with different stages of CRC (n = 98) were included in the study.
All patients have been diagnosed for colorectal adenocarcinoma by pathology examination, 45 patients with
colon carcinoma and 53 with rectosigmoid cancer. A size-based separation method (MetaCell®) for viable CTC
enrichment from peripheral blood was used to assess the presence of CTCs by cytomorphological evaluation
using vital fluorescence microscopy.

Results. Cytomorphological analysis revealed that 81 (83%) tested samples were CTC-positive and 17 (17%)
were CTC-negative. We report a successful isolation of CTCs with proliferation potential in patients with CRC.
The CTCs were cultured *in vitro* for further downstream applications. Some of the isolated CTCs were able to
grow *in vitro* for 6 months as a standard cell culture.

Conclusions. We established a reliable, inexpensive and relatively fast protocol for CTCs enrichment in CRC
patients by means of vital fluorescence staining which enables their further analysis *in vitro*.
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Key words: circulating tumor cells; colon cancer; rectosigmoid cancer; staging; cell culture; MetaCell®

Introduction

An idea of a minimally invasive way to obtain accu-
rate information on tumors from a blood sample,
also known as liquid biopsy, has gained increasing

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attention in cancer diagnosis, risk stratification and
monitoring treatment response. Liquid biopsies are
noninvasive tests using blood or bodily fluids to detect
circulating tumor cells (CTCs) or the products of
tumor cells, such as fragments of nucleotides or pro-
teins that are shed into biological fluids from primary
or metastatic tumors [1]. The analysis of published
clinical studies provides coherent evidence that the
presence of CTCs detected in peripheral blood is
a strong prognostic factor in patients with colorectal
carcinoma (CRC) [1–3].

The majority of the studies used immunomagnetic methods or RT-PCR for CTCs detection [1, 3–5]. These methods are dependent on specification of separated CTCs, especially, their cell surface antigens such as epithelial cell adhesion molecule (EPCAM) or they rely on a specific targeting of RNA sequences. This seems problematic for these methods because one of the key aspects that emerged from the analysis of CTCs is their remarkable heterogeneity, both considering the expression of specific cancer-associated markers, and also their phenotypic characteristics such as tumor-seeding potential. Therefore, we have taken a different approach based on the implementation of cell-size separation protocol of CTCs in CRC patients. This study presents first data of the application of this method to determine the occurrence of CTCs in the differently staged CRC patients.

Material and methods

Patients. To date 98 patients with diagnosed CRC have been enrolled into the study in accordance with the Declaration of Helsinki. All patients were candidates for surgery or surgical diagnosis. Based on the informed consent, clinical data were collected from all patients. For each patient, approximately 18 mL of venous blood was drawn from the antecubital vein and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL of blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the blood draw.

CTCs enrichment and culture. A size-based separation method for viable CTC enrichment from peripheral blood has recently been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) [6, 7]. The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (with pores of 8 μm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL with physiological fluid. The standard 8 mL peripheral blood sample from patients suffering from CRC was transferred into the filtration tube. Detailed separation protocol has been described previously [8–10]. The CTCs were grown in RPMI medium with 10% fetal blood serum (FBS, both purchased from Sigma-Aldrich, St. Louis, MO, USA) for a minimum of 3 days on the membrane. Alternatively, the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be transferred on a microscopic slide. Microscopic slide is preferred if cytological analysis is planned. If an intermediate analysis of CTCs is awaited, the CTCs fraction is transferred in phosphate-buffered

saline (PBS, 1.5 mL) to a cytospin slide. The slide is then dried for 24 h and analyzed by cytology (May-Grünwald staining) and/or by automated immunocytology protocols (Ventana, Benchmark Ultra, Roche, Tucson, AZ, USA) using standard differential diagnostic antibodies for the pathology evaluation process.

Cytomorphological analysis. After 3–5 days of culture, nucleus and cytoplasm of viable cells were stained by vital fluorescent dyes Nucblue® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and Celltracker™ Green CMF-DA Dye (Thermo Fisher Scientific), respectively. Staining procedures followed manufacturer protocols.

The stained fixed cells captured on the membrane were examined using light microscopy in two steps: (i) screening at ×20/×40 magnification to locate the cells; (ii) observation at ×40/×60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, and their digital images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size $\geq 10 \mu$m); (ii) irregular nuclear contour; (iii) visible cytoplasm, cells size over 15 μm; (iv) prominent nucleoli; (v) high nuclear-cytoplasmic ratio; (vi) presence of proliferating cell, (vii) actively invading cells creating 2D or 3D cell groups.

Cell cultures of CTCs obtained from colorectal cancer patients. Membrane with captured cells was washed with RPMI medium and transferred into culture plate. Four mL of RPMI medium supplemented with 10% FBS, amphotericin B (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich) were added to the culture medium. Captured cells were cultured *in vitro* under standard conditions (37°C, 5% CO_2) for 3–5 days.

Statistical analysis. The Chi-Square test was used to determine if there is a significant relationship between two nominal (categorical) variables (*e.g.* metastasis presence and CTC-positivity). Contingency tables in order to decide whether or not effects are present were analyzed (http://vassarstats.net/newcs.html). Statistical significance of differences between two compared groups and CTC presence was set at $p < 0.05$.

Results

Patients' characteristics
Patients diagnosed with different stages of CRC (n = 98, 61 male and 37 female patients) were included in the study (Table 1). The median age was 61.7 years (range 38–90 years). All patients were diagnosed with colorectal adenocarcinoma by pathology examination, 45 patients presented with

Table 1. Clinical-pathological characteristics of the colorectal cancer patients' cohort and the occurrence of circulating tumor cells (CTCs) in blood

Patients	n	Positive CTC	%	Negative CTC	%	p
Colon cancer	45	40	88.89	5	11.11	n.s.
Rectosigmoid cancer	53	41	77.36	12	22.64	n.s.
Tumor size		Positive CTC		Negative CTC		
T1	6	5	83.33	1	16.67	n.s.
T2	19	13	68.42	6	31.58	n.s.
T3	52	44	84.61	8	15.38	n.s.
T4	21	19	90.48	2	9.52	n.s.
Lymph node involvement		Positive CTC		Negative CTC		
N0	52	43	82.69	9	17.31	n.s.
N1	20	17	85.00	3	15.00	n.s.
N2	26	21	80.77	5	19.23	n.s.
Metastasis		Positive CTC		Negative CTC		
M0	67	54	80.60	13	19.40	n.s.
M1	31	27	87.10	4	12.90	n.s.
Grade		Positive CTC		Negative CTC		
G1	3	3	100.00	0	0	n.s.
G2	63	50	79.37	13	20.63	n.s.
G3	32	28	87.50	4	12.50	n.s.
Stage		Positive CTC		Negative CTC		
I	18	14	77.78	4	22.22	n.s.
II	28	26	92.86	2	7.14	n.s.
III	18	13	72.22	5	27.78	n.s.
IV	33	28	84.85	5	15.15	n.s.

The characteristics of the patients' cohort and CTC occurrence was compared by chi-square test. At $p \geq 0.05$ the comparison showed no significant (n.s.) difference for the tested subgroups.

colon carcinoma and 53 with rectosigmoid cancer (Table 1).

Cytomorphological analysis revealed that 81 (83%) of all CRC patients were CTC-positive in peripheral blood. Into study were enrolled 45 patients with colon cancer of which 40 (89%) were CTC-positive and 53 patients with rectosigmoid cancer of which 41 (77%) patients were CTC-positive (Table 1).

The presence of CTCs in different stages of colorectal cancer according to TNM classification

The frequency of the CTC positivity is reported for different patient subgroups in relationship to tumor size, lymph node involvement and distant metastases. The CTC positivity for different disease stages and tumor grade subgroups is summarized in Table 1. Patients were CTC positive in majority of all TNM stages, and disease and grade stages (Table 1).

Cultures of CTCs isolated from blood of colorectal cancer patients

We report a successful isolation of CTCs with proliferation potential in patients with CRC. The cells captured by size-based filtration approach showed a viable condition, which enabled setting up cultures of CTCs which viability was unaffected by antibodies (*e.g.* anti-EPCAM antibodies) or lysing solutions (*e.g.* erythrocyte lysing solution). The CTCs were cultured *in vitro* for further downstream applications (Fig. 1). Some of the isolated CTCs were grown *in vitro* for as long as 6 months as a standard cell culture.

Discussion

The classification of tumor (pTNM staging) and the residual tumor status following treatment are the strongest predictors of outcome for patients with CRC. A careful pTNM classification enables an accurate estimation of prognosis; therefore, it can be
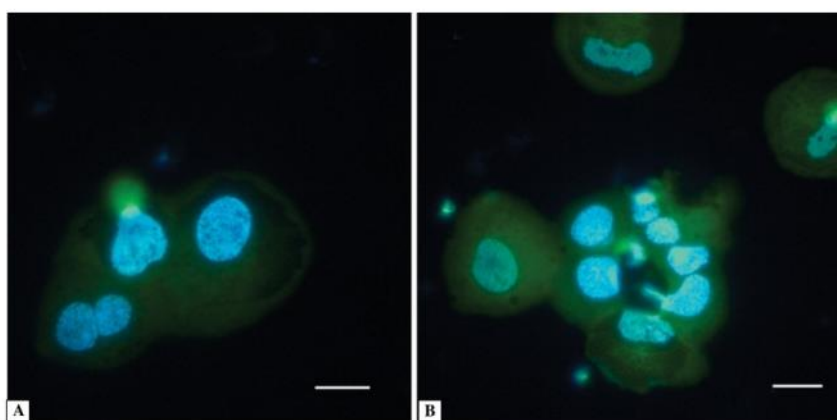
Figure 1. Morphology of CTCs isolated from patients with CRC. **A.** CTCs captured on the separation membrane; **B.** CTCs after successful short term *in vitro* cultivation — 5 days after isolation. Cells were visualized by vital fluorescent staining with NucBlue® and Celltracker® as described in Material and methods. Bars represent 10 μm.

considered the gold standard for analyzing the results of any treatment [11].

Similarly, CTCs enumeration has been established as a prognostic marker for CRC [1, 12–14]. In a large meta-analysis including 12 studies between 1998 and 2011, the presence of CTCs in patients with metastatic colorectal cancer (mCRC) correlated with shorter progression-free survival (PFS) and overall survival (OS) [14].

Matsusaka *et al.* showed that patients with ≥ 3 CTCs in 7.5 mL blood at 2 and 8–12 weeks after initiation of chemotherapy had shorter median progression-free survival (PFS) and overall survival (OS) than patients with < 3 CTC counts. Patients with CTC counts ≥ 3 at baseline with a decrease in the CTC count to < 3 during treatment had a median PFS that was similar to patients with persistently low CTC counts. They concluded that a decrease in CTC count to < 3 at 2 weeks after initiating chemotherapy was an indicator of treatment efficacy [15].

The use of different methodologies for CTC detection has shown conflicting results, and the lack of a standardized technology complicates the implementation of CTCs examination in routine clinical practice. In addition, significant differences in CTC detection rates among the molecular methods of their detection have been reported [16–18].

In comparison with other studies [1–3, 12–15] the detection rate of CTCs in the present study seems high. However, a recent study of breast cancer showed that tumor cells can leave the primary site very early during tumor progression and evolve independently at the metastatic site [19, 20]. The genetic analyses

showed that 80% of metastases were derived from early disseminated tumor cells [20].

CTCs can be used for longitudinal molecular and genetic analyses of the tumor and may aid in targeted therapy investigations. CRC patients with CTCs carrying wild-type KRAS show longer PFS and OS when treated with chemotherapeutics and cetuximab [21]. The detection of KRAS mutation in CTCs from peripheral blood may predict the response to cetuximab plus chemotherapy in CRC patients [21]. These findings indicate that the detection of KRAS mutational status in CTCs by using gene expression array has potential clinical applications for selecting CRC patients who may benefit from cetuximab therapy.

CTCs detection and characterization may be a valuable tool to refine prognosis and CTCs can be predictive biomarkers in treatment of colorectal cancer.

Here we presented high accordance of the TNM stages of CRC with the occurrence of CTCs in patient's blood detected by a reliable, inexpensive and relatively fast method for CTCs enrichment and evaluation based on cytomorphological analysis by means of vital fluorescence staining.

References

1. Rahbari NN, Aigner M, Thorlund K, et al. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. Gastroenterology. 2010; 138(5): 1714–1726, doi: 10.1053/j.gastro.2010.01.008, indexed in Pubmed: 20100481.
2. Liberko M, Kolostova K, Bobek V. Essentials of circulating tumor cells for clinical research and practice. Crit Rev Oncol Hematol. 2013; 88(2): 338–356, doi: 10.1016/j.critrevonc.2013.05.002, indexed in Pubmed: 23830807.

3. Eliasova P, Kolostova K, Kobierzycki C, et al. Clinical studies monitoring circulating and disseminated tumor cells in gastrointestinal cancers. Folia Histochem Cytobiol. 2013; 51(4): 265–277, doi: 10.5603/FHC.2013.0037, indexed in Pubmed: 24497131.

4. Huang MY, Tsai HL, Huang JJ, et al. Clinical Implications and Future Perspectives of Circulating Tumor Cells and Biomarkers in Clinical Outcomes of Colorectal Cancer. Transl Oncol. 2016; 9(4): 340–347, doi: 10.1016/j.tranon.2016.06.006, indexed in Pubmed: 27567958.

5. Tan C, Zhou L, El-Deiry W. Circulating tumor cells versus circulating tumor DNA in colorectal cancer: pros and cons. Curr Colorectal Cancer Rep. 2016; 12(3): 151–161, doi: 10.1007/s11888-016-0320-y, indexed in Pubmed: 27516729.

6. Kolostova K, Matkowski R, Jędryka M, et al. The added value of circulating tumor cells examination in ovarian cancer staging. Am J Cancer Res. 2015; 5(11): 3363–3375, indexed in Pubmed: 26807317.

7. Kolostova K, Spicka J, Matkowski R, et al. Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers. Am J Transl Res. 2015; 7(7): 1203–1213, indexed in Pubmed: 26328005.

8. Cegan M, Kolostova K, Matkowski R, et al. In vitro culturing of viable circulating tumor cells of urinary bladder cancer. Int J Clin Exp Pathol. 2014; 7(10): 7164–7171, indexed in Pubmed: 25400813.

9. Bobek V, Matkowski R, Gürlich R, et al. Cultivation of circulating tumor cells in esophageal cancer. Folia Histochem Cytobiol. 2014; 52(3): 171–177, doi: 10.5603/FHC.2014.0020, indexed in Pubmed: 25308732.

10. Kolostova K, Zhang Y, Hoffman RM, et al. In vitro culture and characterization of human lung cancer circulating tumor cells isolated by size exclusion from an orthotopic nude-mouse model expressing red fluorescent protein. J Fluoresc. 2014; 24(5): 1531–1536, doi: 10.1007/s10895-014-1439-3, indexed in Pubmed: 25141982.

11. Hermanek P. pTNM and residual tumor classifications: problems of assessment and prognostic significance. World J Surg. 1995; 19(2): 184–190, indexed in Pubmed: 7754621.

12. Cohen SJ, Punt CJA, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol. 2008; 26(19): 3213–3221, doi: 10.1200/JCO.2007.15.8923, indexed in Pubmed: 18591556.

13. Cohen SJ, Punt CJA, Iannotti N, et al. Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. Ann Oncol. 2009; 20(7): 1223–1229, doi: 10.1093/annonc/mdn786, indexed in Pubmed: 19282466.

14. Groot Koerkamp B, Rahbari NN, Büchler MW, et al. Circulating tumor cells and prognosis of patients with resectable colorectal liver metastases or widespread metastatic colorectal cancer: a meta-analysis. Ann Surg Oncol. 2013; 20(7): 2156–2165, doi: 10.1245/s10434-013-2907-8, indexed in Pubmed: 23456317.

15. Matsusaka S, Suenaga M, Mishima Y, et al. Circulating tumor cells as a surrogate marker for determining response to chemotherapy in Japanese patients with metastatic colorectal cancer. Cancer Sci. 2011; 102(6): 1188–1192, doi: 10.1111/j.1349-7006.2011.01926.x, indexed in Pubmed: 21401804.

16. Schuster R, Max N, Mann B, et al. Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers. Int J Cancer. 2004; 108(2): 219–227, doi: 10.1002/ijc.11547, indexed in Pubmed: 14639606.

17. Sher YP, Shih JY, Yang PC, et al. Prognosis of non-small cell lung cancer patients by detecting circulating cancer cells in the peripheral blood with multiple marker genes. Clin Cancer Res. 2005; 11(1): 173–179, indexed in Pubmed: 15671543.

18. Oberg ANV, Lindmark GE, Israelsson ACE, et al. Detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR for CEA and CK20 mRNAS. Int J Cancer. 2004; 111(1): 101–110, doi: 10.1002/ijc.20231, indexed in Pubmed: 15185350.

19. Ghajar CM, Bissell MJ. Metastasis: Pathways of parallel progression. Nature. 2016; 540: 528–529, doi: 10.1038/nature21104, indexed in Pubmed: 27974802.

20. Hosseini H, Obradović MMS, Hoffmann M, et al. Early dissemination seeds metastasis in breast cancer. Nature. 2016; 540: 552–558, doi: 10.1038/nature20785, indexed in Pubmed: 27974799.

21. Yen LC, Yeh YS, Chen CW, et al. Detection of KRAS oncogene in peripheral blood as a predictor of the response to cetuximab plus chemotherapy in patients with metastatic colorectal cancer. Clin Cancer Res. 2009; 15(13): 4508–4513, doi: 10.1158/1078-0432.CCR-08-3179, indexed in Pubmed: 19549774.

JAKABOVA A., BIELCIKOVA Z., POSPISILOVA E., MATKOWSKI R., SZYNGLAREWICZ B., STASZEK-SZEWCZYK U., ZEMANOVA M., PETRUZELKA L., ELIASOVA P., KOLOSTOVA K., BOBEK V.; Molecular characterization and heterogeneity of circulating tumor cells in breast cancer. *Breast Cancer Res Treat*. 2017 Dec;166(3):695-700. ISSN: 0167-6806 (Print) 1573-7217 (Electronic). DOI: 10.1007/s10549-017-4452-9. IF: **3.626/2016.**

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PRECLINICAL STUDY

Molecular characterization and heterogeneity of circulating tumor cells in breast cancer

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Abstract

Introduction This study analyzes peripheral blood samples from breast cancer (BC) patients. CTCs from peripheral blood were enriched by size-based separation and were then cultivated in vitro. The primary aim of this study was to demonstrate the antigen independent CTC separation method with high CTC recovery. Subsequently, CTCs enriched several times during the treatment were characterized molecularly.

Methods Patients with different stages of BC ($N = 167$) were included into the study. All patients were candidates for surgery, surgical diagnostics, or were undergoing chemotherapy. In parallel, 20 patients were monitored regularly and in addition to CTC presence, also CTC character was examined by qPCR, with special focus on HER2 and ESR status.

Results CTC positivity in the cohort was 76%. There was no significant difference between the tested groups, but the highest CTC occurrence was identified in the group undergoing surgery and similarly in the group before the start of neoadjuvant treatment. On the other hand, the lowest CTC frequencies were observed in the menopausal patient group (56%), ESR+ patient group (60%), and DCIS group (44.4%). It is worth noting that after completion of neoadjuvant therapy (NACT) CTCs were present in 77.7% of cases. On the other hand, patients under hormonal treatment were CTC positive only in 52% of cases.

Discussions Interestingly, HER2 and ESR status of CTCs differs from the status of primary tumor. In 50% of patients HER2 status on CTCs changed not only from HER2+ to HER2−, but also from HER2− to HER2+ (33%). ESR status in CTCs changed only in one direction from ESR+ to ESR−.

Conclusions Data obtained from the present study suggest that BC is a heterogeneous disease but CTCs may be detected independently of the disease characteristics in 76% of patients at any time point during the course of the disease. This relatively high CTC occurrence in BC should be considered when planning the long-term patient monitoring.

Keywords CTCs · Circulating tumor cells · Breast cancer · Cultivation · In vitro · MetaCell · Gene expression

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Introduction

Enumeration of circulating tumor cells (CTCs) has showed a prognostic role in various stages of the breast cancer (BC). Hormone receptors (estrogen and progesterone) and HER2 status of primary BC tumor have been established during standard clinical biopsies and are of crucial importance in the choice of treatment. Real-time tumor monitoring through CTC enumeration could be an important indicator of individual cancer development [1].

CTCs as biomarkers can offer some valuable information about a patient's tumor, if detection, separation, and characterization are performed in a reliable manner. Although occurrence of CTCs in patients' peripheral blood is often very low, enrichment methods can be introduced for CTC separation before their characterization. They are usually based on surface protein expression, size, density, electric charges, or deformability of CTCs.

This study analyzes peripheral blood samples from patients with BC. CTCs from peripheral blood were enriched by size-based separation and then cultivated in vitro. The primary aim of this study was to demonstrate the antigen independent high sensitive separation method and a possibility of molecular characterization of CTCs enriched several times during the treatment.

Materials and methods

Patients

To date 167 patients with diagnosed BC have been enrolled in the study in accordance with the Declaration of Helsinki. All patients were candidates for surgery, surgical diagnostics, or with planned or applied chemotherapy. Based on their informed consent, clinical data were collected from all participating patients. Basic cytopathological data are reported in Table 1. For each patient, approximately 2×8 mL of venous blood was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 h after the blood draw.

CTCs enrichment and culture

The recently introduced size-based separation method for viable CTC enrichment process (MetaCell®, MetaCell s.r. o., Ostrava, Czech Republic) [2–6] is based on the filtration of peripheral blood through a porous polycarbonate

Table 1 Basic cytopathological characteristics of patients

	N	(%)
Stage		
0	3	2
IA	45	30
IIA	64	42.7
IIB	20	13.3
IIIA	13	8.7
IIIB	1	0.67
IIIC	4	2.67
Histopathological features		
Benign	2	1.7
DCIS	9	7.6
LCIS	1	0.85
IDC (NST)	76	65.6
ILC	14	11.86
Mixed	16	13.6
Menopausal status		
Premenopausal	65	39.39
Menopausal	18	10.9
Postmenopausal	82	49.7
Tumor size		
T1	63	61.1
T2	36	34.9
T3	4	3.8
Nodal involvement		
N0	56	56.5
N1	37	37.3
N2	6	6
Grading		
G1	7	11.8
G2	24	40.6
G3	28	47.4
HR and HER2 status		
HR+ HER2+	16	11.7
HR− HER2+	7	5.1
HR+ HER2−	91	66.4
HR− HER2−	23	16.8

membrane (with pores of 8 μm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL of fluid. The standard 8 mL peripheral blood sample from patients suffering from BC was transferred into the filtration tube. Gradual transfer of the blood in several steps is preferred to prevent blood clotting on the membrane filter. The peripheral blood flow is supported by capillary action of the absorbent touching the membrane filter. The filtered CTCs were observed immediately after filtration on the membrane. The control and presence of filtered CTCs immediately after isolation eliminates false negative results. The membrane filter is

kept in a plastic ring that is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and CTCs are cultured on the membrane in vitro under standard cell-culture conditions (37 °C, 5% atmospheric CO_2) and observed by inverted microscope. The CTCs were grown in FBS-enriched RPMI medium (10%) for a minimum of 14 days on the membrane. Alternatively, the enriched CTC fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be translocated on a microscopic slide. Microscopic slide is preferred if immunohistochemistry/immunofluorescence analysis is planned. If an immediate CTC analysis is awaited, the CTC fraction is transferred in PBS (1.5 mL) to a cytospin slide. The slide is then dried for 24 h and analyzed by histochemistry (May-Grünwald staining) and/or by automated immunohistochemistry protocols (Ventana, Benchmark Ultra, Roche) using standard differential diagnostic antibodies in the pathological evaluation process.

Cytomorphological analysis

The stained fixed cells captured on the membrane were examined using light microscopy in two steps: (i) screening at ×20 magnification to locate the cells; (ii) observation at ×40/×60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized, and the images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size ≥10 μm); (ii) irregular nuclear contour; (iii) visible cytoplasm, cells size over 15 μm; (iv) prominent nucleoli; (v) high nuclear-cytoplasmic ratio; (vi) proliferation; (vii) actively invading cells creating 2D or 3D cell groups.

Gene expression analysis (GEA)

The key purpose of GEA was to compare gene expression of tumor-associated markers in the CTC-enriched fractions to that in the whole blood (white blood cells). Gene expression analysis can be performed to confirm the origin of the captured cells on the separation membrane. Gene expression analysis (GEA) allows up to 20 tumor-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostics markers for qPCR test are chosen in accordance with the expected diagnosis.

RNA is isolated from the whole blood and CTC-enriched fraction on the membrane. The CTC-enriched fraction of cells grown on the separation membrane in vitro (the so-called "membrane fraction") was used for RNA isolation.

Finally, CTC-gene expression analysis allows identification of the relative amount of tumor-associated (TA) markers in the whole blood and in CTC-enriched fractions. If the tumor-associated genes are highly expressed in the CTC fraction, a subsequent analysis of chemoresistance-associated (CA) genes is performed. Molecular analysis helps to identify which type of chemotherapeutic agents may be of use in tumor therapy and assigned as personalized cancer therapy based on CTC.

The cells captured on the membrane are lysed by RLT-buffer with beta-mercapto-ethanol (Qiagen). RNA is then isolated using the RNeasy Mini Kit (Qiagen). RNA from the whole blood is isolated with a modified procedure and the quality/concentration of RNA is measured by Nano-Drop (ThermoScientific). As there are only up to a few hundred cells on the membrane, the median concentration of RNA is quite low (5–10 ng/μl). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA production. Gene expression analysis was performed using Taqman chemistry with Taqman MGB-probes for all the tested genes (Life Technologies).

The following genes associated with tumorigenic character and therapeutic potential in breast cancer were chosen for the multimarker GEA panel: ACTIN, CD45, CD68, EPCAM, MUC1, KRT18, KRT19, ESR, PGR, MAMMAGLOBIN, HER2, CD24, CD44. Additionally, genes associated with chemoresistance were tested (MRP1-10, MDR1, ERCC1).

Statistical analysis

All analyses were performed using clinicopathological information transformed into variables 0 and 1 if applicable for tested characteristics. Chi squared test, t tests, cluster analysis, and correlation analysis of qPCR data were outperformed using GeneX (MultiD, SE) and GraphPadPrism versus 5 (Graphpad, US). P value of less than 0.05 was considered statistically significant.

Results

The main focus of the study was to detect CTCs shortage in BC patients by a new methodological approach which is based on size-dependent separation of CTCs and subsequent cytomorphological evaluation. Cytomorphological evaluation using vital fluorescence microscopy approach (Fig. 1) enables further use of the viable captured cells for RNA/DNA analysis.

Patients diagnosed with different stages of breast cancer (BC) ($N = 167$) were included into the study. The patients were divided based on clinicopathological criteria and CTC presence was tested. Summary of the collected CTC positivity data is presented in Table 2.
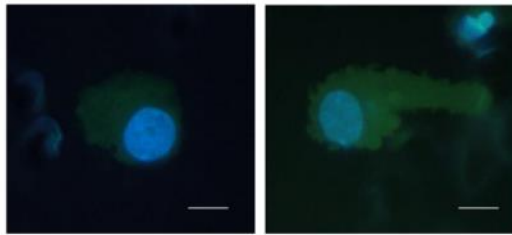
Fig. 1 CTCs isolated from a patient with breast cancer, captured on the separation membrane (vital fluorescent staining— NucBlue® and Celltracker®). *Bar* represents 10 μm

CTC positivity in tested cohort was 76%. There was no significant difference between tested subgroups, identifying a possible CTC presence, but the highest CTC occurrence was observed in the group undergoing surgery (86.6%) and similarly in the group before the start of neoadjuvant and adjuvant treatment (82.3%).

It is important to comment on relatively high CTCs presence even after neoadjuvant therapy has been completed (77.7%). It can be assumed that in these patients therapy did not diminished all the cancer cell types.

There were no significant differences in CTC frequencies observed based on stage definitions. Considering the histopathological character of the primary tumor, the lowest CTC positivity was observed in DCIS (44.4%). Relatively low CTC frequency was observed in the menopausal patient group (55.5%).

Furthermore, it can be concluded that in tumors with ESR expression (ESR+) and without PGR expression (PGR-) CTCs were detected only in 60% (9/15) of tested cases, whereas in ESR+/PGR+ tumors CTC positivity was 73% (68/93). On the other hand, in patients with ESR-negative tumors CTCs were detected in 96.7% which is almost all of the patients under study (30/31). Therefore, it must be mentioned that during the therapy only 52.9% (9/17) of patients exhibited CTCs. Nevertheless, menopausal stage has to be considered if ESR/PGR expression is evaluated. The correlation of the menopausal status and ESR/PGR expression is illustrated in Fig. 2 which shows that hormonal receptor-positive tumors exhibit the lowest CTC detection frequencies in comparison to the HR-groups.

Similarly, even if not statistically significant, it can be seen that HR +/HER2− tumors, irrespective of the menopausal stage show the lowest CTC frequency rates (see Fig. 3).

In parallel, 20 patients were monitored regularly during the course of the disease and in addition to CTC presence, CTCs character was also examined by qPCR with special focus on HER2 and ESR status. In total 43 qPCR analysis

were evaluated. Therapeutically, the most relevant findings are as follows: HER2 and ESR status of CTCs may differ from the status of primary tumor.

Table 2 CTC positivity identified in BC—patient subgroups

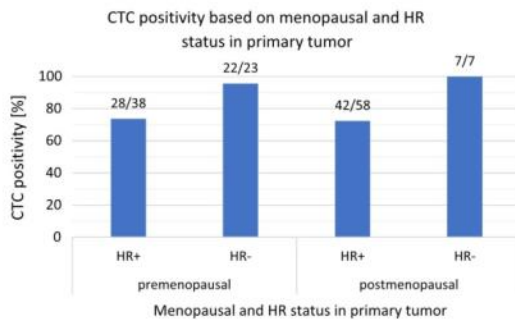|	N	(%)
CTC Positivity	CTC+	
CTC+	**119**	**72.1**
CTC−	**46**	**27.9**
Stage		
0	3	100
IA	31	68.9
IIA	47	73.4
IIB	16	80
IIIA	10	76.9
IIIB	0	0
IIIC	4	100
Histopathological features		
DCIS	4	**44.4**
LCIS	1	100
IDC (NST)	55	72.4
ILC	13	92.9
Mixed	9	56.3
Menopausal status		
Premenopausal	51	78.4
Menopausal	10	**55.5**
Postmenopausal	58	70.7
Tumor size		
T1	63	74.6
T2	36	88.8
T3	4	75
Nodal involvement		
N0	23	82
N1	17	78
N2	2	66
Grading		
G1	**5**	70
G2	14	58
G3	23	82
HR and HER2 status		
HR+ HER2+	13	81.3
HR− HER2+	7	100
HR+ HER2−	64	**70.3**
HR− HER2−	22	95.7
ESR+ PGR+ vs. ESR+ PGR−		
Therapy		
Before therapy	28	82.3
During HT	9	**52.9**
After NACT	7	77.7
Before surgery (after biopsy)	39	86.6

Fig. 2 CTC positivity in relation to menopausal stage and primary tumor HR− expression
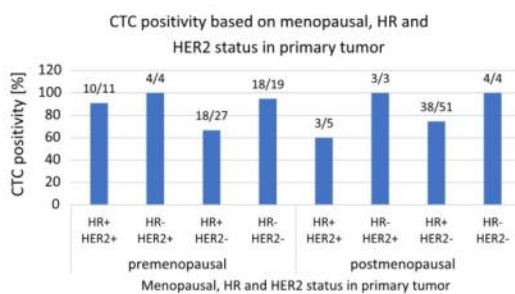

Fig. 3 CTC positivity in relation to menopausal stage and primary tumor HR and HER2− expression

The most frequent changes were seen in the triple negative BC (TNBC) group ($N = 12$) where 27 samples were evaluated. HER2 presence was confirmed in CTCs in four cases, which means that the change from HER2− to HER2+ occurred in 15% of tested samples, but that these four changes can be ascribed to four different patients. The change was relevant for four out of 12 patients (33.3%) which is already a significant number. Similarly, in 50% of patients, HER2 status changed from HER2+ to HER2− (3/6).

ESR status in CTCs changed only in one direction from ESR+ to ESR− (3/3). These patients' primary tumors were diagnosed as ESR+/PGR+/HER2−. This group of patients will most probably exhibit very frequent changes.

Taken together, due to relatively high numbers of CTC positivity in different patient groups, we may conclude that a certain number of CTCs are always present in the blood of the patients. The cells have to be under selection pressure of treatment uninterruptedly. As soon as the selection pressure is stopped, new gene expression profile is displayed by CTCs.

The data obtained in the present study suggest that BC is a heterogeneous disease, but CTCs may be detected independently of the disease characteristics in 76% (119/165) of patients at any time point of the course of the disease.

This relatively high CTC occurrence in BC should be considered in planning the long-term patient monitoring.

Discussion

Treatment decisions in BC are based on the characteristics of the primary tumor without considering the character of minimal residual disease or metastasis. However, tumors are evolving entities and genetic heterogeneity has been detected comparing the primary tumor with subsequent recurrences and metastases and analyzing different regions of the same tumor [7]. It has been hypothesized that the success of personalized treatments greatly depends on the capability to capture and monitor tumor heterogeneity over time and to consequently modulate therapies [8].

Detection and characterization of CTCs can contribute to the understanding of the disease and improved therapy monitoring as well as personalized treatment options. The key step is sensitive isolation and detection of CTCs. To date, various approaches have been also used to visually identify CTCs; however, the techniques employed to perform cell enrichment, immunohistochemical detection, and image analysis are complicated [9, 10]. Moreover, epithelial markers are currently used to detect CTCs; tumor cells, however, may lose their epithelial features during metastasis/dissemination or may not express these markers because of their heterogeneity [11]. Therefore, some CTCs could be unidentified during epithelial-mesenchymal transition (EMT) by the common CTC-enrichment strategies relying on epithelial markers [12]. According to recent findings, more invasive CTCs may lose their epithelial antigens as a result of the EMT process [13] and EMT has been increasingly recognized as the key mechanism of cancer drug resistance [14].

We have used a simple method, without any complicated processing steps, for detecting viable human CTCs in the peripheral blood by using physical features of CTCs. We believe that viable CTCs may be a less invasive, repeatable biomarker for monitoring tumor responses.

In our study more than 76% patients were CTC positive. This result provides evidence that BC cells migrate and disseminate from morphologically very early lesions. Hosseini et al. demonstrated that metastatic dissemination often occurs early during tumor formation [15]. Disseminated cancer cells detected in patients before the manifestation of breast-cancer metastasis contain fewer genetic abnormalities than primary tumors and indicate that dissemination occurs during early stages of tumor growth [16–19].

As demonstrated by the SWOG S0500 trial, the simple enumeration of CTCs is not sufficient to guide therapy [20]. There is increasing evidence that cancer evolves over

time because of its genomic instability and under the selection pressure of systemic treatments. These changes can be responsible for the appearance of drug-resistant clones. In studies of metastatic breast cancer (MBC), a discrepancy was observed between metastases or CTCs and the primary tumors in terms of HER2, estrogen and progesterone receptor expression [21, 22]. The loss of progesterone or estrogen hormone receptor expression in CTCs was described in 40% of receptor-positive MBC, while increased hormone receptor expression was detected in only 8% of triple negative MBC [21].

The clinical use of new CTC detection technique and the molecular characterization of isolated CTCs may lead to the development of personalized anticancer strategy in near future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Banys-Paluchowski M, Krawczyk N, Fehm T (2016) Potential role of circulating tumor cell detection and monitoring in breast cancer: a review of current evidence. Front Oncol 6:255
2. Kolostova K, Matkowski R, Jędryka M, Soter K, Cegan M, Pinkas M, Jakabova A, Pavlasek J, Spicka J, Bobek V (2015) The added value of circulating tumor cells examination in ovarian cancer staging. Am J Cancer Res 5:3363–3375
3. Kolostova K, Spicka J, Matkowski R, Bobek V (2015) Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers. Am J Transl Res 7:1203–1213
4. Kolostova K, Pinkas M, Jakabova A, Pospisilova E, Svobodova P, Spicka J, Cegan M, Matkowski R, Bobek V (2016) Molecular characterization of circulating tumor cells in ovarian cancer. Am J Cancer Res. 6(5):973–980
5. Kolostova K, Cegan M, Bobek V (2014) Circulating tumor cells in patients with urothelial tumors: enrichment and in vitro culture. CUAJ 8(9–10):E715
6. Kolostova K, Zhang Y, Hoffman RM, Bobek V (2014) In vitro culture and characterization of human lung cancer circulating tumor cells isolated by size exclusion from an orthotopic nude-mouse model expressing red fluorescent protein. J Fluoresc 24(5):1531–1536
7. Siu LL (2013) Accounting for tumor heterogeneity in the development of predictive biomarkers. Clin Adv Hematol Oncol 11:312–313
8. Gagan J, van Allen EM (2015) Next-generation sequencing to guide cancer therapy. Genome Med 7:80
9. Eliasova P, Kolostova K, Kobierzycki C, Bobek V (2013) Clinical studies monitoring circulating and disseminated tumor cells in gastrointestinal cancers. Folia Histochem Cytobiol 51(4):265–277
10. Liberko M, Kolostova K, Bobek V (2013) Essentials of circulating tumor cells for clinical research and practice. Crit Rev Hematol Oncol 88(2):338–356
11. Willipinski-Stapelfeldt B et al (2005) Changes in cytoskeletal protein composition indicative of an epithelial-mesenchymal transition in human micrometastatic and primary breast carcinoma cells. Clin Cancer Res 11:8006–8014
12. Mego M, De Giorgi U, Dawood S, Wang X, Valero V, Andreopoulou E, Handy B, Ueno NT, Reuben JM, Cristofanilli M (2011) Characterization of metastatic breast cancer patients with nondetectable circulating tumor cells. Int J Cancer 129:417–423
13. Forte VA, Barrak DK, Elhodaky M, Tung L, Snow A, Lang JE (2016) The potential for liquid biopsies in the precision medical treatment of breast cancer. Cancer Biol. Med. 13:19–40
14. Du B, Shim JS (2016) Targeting epithelial-mesenchymal transition (emt) to overcome drug resistance in cancer. Molecules 21:7
15. Hosseini H et al (2016) Early dissemination seeds metastasis in breast cancer. Nature. doi:10.1038/nature20785
16. Schardt JA et al (2005) Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer. Cancer Cell 8:227–239
17. Schmidt-Kittler O et al (2003) From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. Proc Natl Acad Sci USA 100:7737–7742
18. Hüsemann Y et al (2008) Systemic spread is an early step in breast cancer. Cancer Cell 13:58–68
19. Sänger N et al (2011) Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. Int J Cancer 129:2522–2526
20. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, Tejwani S, Schott AF, O'Rourke MA, Lew DL et al (2014) Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 32:3483–3489
21. Aktas B, Kasimir-Bauer S, Muller V, Janni W, Fehm T, Wallwiener D, Pantel K, Tewes M (2016) DETECT Study Group. Comparison of the HER2, estrogen and progesterone receptor expression profile of primary tumor, metastases and circulating tumor cells in metastatic breast cancer patients. BMC Cancer 16:522
22. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV, Terstappen LW (2006) Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 12:4218–4224

Circulating tumor cells in pancreatic cancer patients: Enrichment and cultivation

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Abstract

AIM: To investigate the feasibility of separation and cultivation of circulating tumor cells (CTCs) in pancreatic cancer (PaC) using a filtration device.

METHODS: In total, 24 PaC patients who were candidates for surgical treatment were enrolled into the study. Peripheral blood samples were collected before an indicated surgery. For each patient, approximately 8 mL of venous blood was drawn from the antecubital veins. A new size-based separation MetaCell® technology was used for enrichment and cultivation of CTCs *in vitro*. (Separated CTCs were cultured on a membrane in FBS enriched RPMI media and observed by inverted microscope. The cultured cells were analyzed by means of histochemistry and immunohistochemistry using the specific antibodies to identify the cell origin.

RESULTS: CTCs were detected in 16 patients (66.7%) of the 24 evaluable patients. The CTC positivity did not reflect the disease stage, tumor size, or lymph node involvement. The same percentage of CTC positivity was observed in the metastatic and non-metastatic patients (66.7% *vs* 66.7%). We report a successful isolation of CTCs in PaC patients capturing proliferating cells. The cells were captured by a capillary action driven size-based filtration approach that enabled cells cultures from the viable CTCs to be unaffected by any antibodies or lysing solutions. The captured cancer cells displayed plasticity which enabled some cells to invade the separating membrane. Further, the cancer cells in the "bottom fraction", may represent a more invasive CTC-fraction. The CTCs were cultured *in vitro* for further downstream applications.

CONCLUSION: The presented size-based filtration method enables culture of CTCs *in vitro* for possible downstream applications.

Key words: Pancreatic cancer; Circulating tumor cells; Biomarker; Cultivation

Core tip: Circulating tumor cells role in the process of pancreatic cancer dissemination should be studied in the context of the disease management. The ability to *in vitro* culture pancreatic circulating tumor cells (CTCs) could potentially help with the development of innovative treatments and diagnostic technologies. We presented simple size-based separation device for the

isolation of viable CTCs. The isolation process is gentle allowing the subsequent CTC-cultivation *in vitro* and is antibody independent.

INTRODUCTION

The lethal nature of cancer is caused by its invasive character, and spread *via* blood and lymphatic system to distant locations generating metastatic disease. Moreover, pancreatic cancer (PaC) counts to the solid tumors with the shortest overall survivals. The aggressiveness of the disease is demonstrated in clinic by very early metastatic disease and chemoresistance[1-3].

Prognostic value of tumor cells disseminated to the blood and bone marrow has been shown for various types of solid tumors. Circulating tumor cells (CTCs) are cells shed from primary tumor and metastatic sites to the peripheral blood.

Large patient series of breast, prostate, lung, colon cancer have been tested for CTCs, but no complete results have been reported so far in pancreatic cancer clinical trials[4-6].

The limitation of the recently available tumor markers in PaC could be overcome by CTC.

The analytical methods developed to identify CTCs in PaC include direct and indirect CTCs- detection. Analytical assays based on antibodies against EpCAM antigen expressed on the cells surface count to the direct CTC-isolation methods together with size based separations. The polymerase chain reaction-based assays analyzing DNA and RNA count for indirect detection methods[7,8].

Characterization of CTCs in PaC including enumeration could be an important part of the diagnostic process. CTCs detection aims to reveal the tumor recurrence risk, chemo and radiotherapy resistance markers[9].

Moreover, the conventional prognostic indicators to predict patient outcome are often imperfect, owing mainly to tumor plasticity and subjective assessment criteria. Therefore, there is an urgent need for the establishment of new sensitive prognostic methods capable of identifying patients with a worse prognosis or those who will progress quickly.

In the present study, we have employed size-based separation method to detect CTCs. Our goal was to create an accurate assay that would improve the both detection and cultivation of CTCs/disseminated tumor cells (DTCs) of pancreatic cancer patients avoiding false-positive results and to allow for the personalization of therapy regimens.

Table 1 Patient characteristics and circulating tumor cells examination results *n* (%)

Patient characteristics	Total patients (*n*)	With detected CTC
T stage	24	
T1	0	0 (0)
T2	2	1 (50)
T3	11	9 (81.8)
T4	11	6 (54.5)
N stage		
N0	7	6 (85.7)
N1	17	10 (58.9)
M stage		
M0	21	14 (66.7)
M1	3	2 (66.7)
Grading		
1	1	0 (0)
2	13	8 (61.5)
3	9	8 (88.9)
4	1	0 (0)
Disease stage		
I	1	1 (100)
II A	5	4 (80)
II B	7	5 (71.4)
III	8	4 (50)
IV	3	2 (66.7)

CTCs: Circulating tumor cells.

MATERIALS AND METHODS

Patients

To date, 24 patients with diagnosed PaC have been enrolled into the study in accordance with Declaration of Helsinki. All patients were candidates for surgery treatment, but 9 out of the 24 patients (37.5%) were seen as inoperable within surgery. Based on the informed consent clinical data were collected from all participating patients. The patient sample characteristics are shown in Table 1. Peripheral blood (PB) was collected prior to surgery. For each patient, peripheral blood (8 mL) was withdrawn into S-Monovette tubes (Sarstedt AG and Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The isolation procedure was completed within 24 h after the blood withdrawal (the samples were stored at 4-8 ℃ up to 24 h).

CTCs enrichment and culture

Recently, a new size based separation method for viable CTC - enrichment from PB has been introduced (Meta-Cell®, MetaCell s.r.o., Ostrava, Czech Republic)[10]. The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (pores with 8 μm diameter). The minimum and maximum volume of the filtered PB may be adjusted up to 50 mL with fluid. The standard 8 mL of PB from patients suffering with PaC was transferred into the filtration tube-set. Successive blood transfer in several steps is preferred to prevent the blood clotting on the membrane filter. The PB flow is driven by capillary action of the absorbent touching the membrane filter. The whole isolation procedure runs at room tempera-
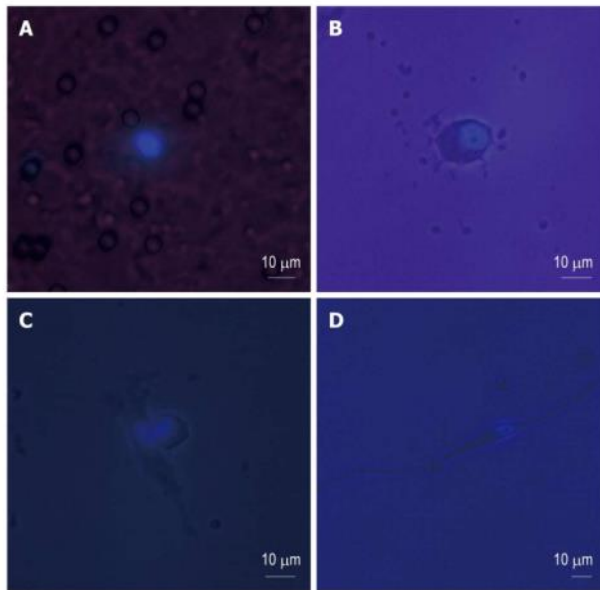
Figure 1 Circulating tumor cells captured and cultured on the membrane filter (A), cultured *in vitro* (A, B, C, D) with visualized nucleoli and nucleus counterstained with DAPI. The circulating tumor cells (CTCs) cultured *in vitro* may grow through the membrane *via* the ability of changing the cell shape (C, D).
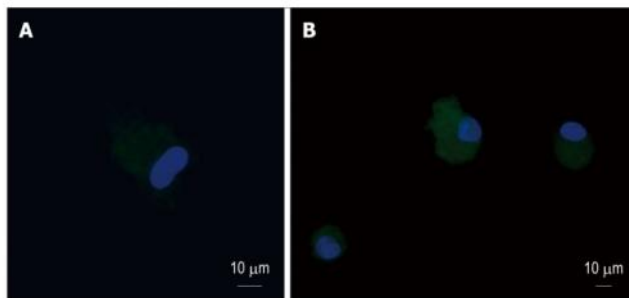
Figure 2 Circulating tumor cells captured and cultured on the membrane filter, incubated with CK-18-FITC antibody, nucleus was counterstained by DAPI (A, B).

ture. The filtered CTCs were observed on the membrane immediately after filtration by light microscopy and subsequently (after 2 h) by fluorescent microscopy using unspecific nuclear stain (NucBlue™, Life Technologies). For some of the tested samples unspecific cytoplasmic stain (CellTracker™, Life Technologies) has been used as well to identify viable CTCs. The fluorescent analysis enables to distinguish cytomorphology of the recently enriched CTC fraction with a very high percentage of sensitivity.

The control of presence of the captured CTCs immediately after the isolation process helps to avoid false negative results of examination.

The membrane filter is kept in a plastic ring that is transferred into the 6-well cultivation plate, 2 mL RPMI media is added to the filter top and 2 mL to the well bottom. CTCs are cultured on the membrane *in vitro* under standard cell culture conditions (37 ℃, 5% atmosphere of CO_2) and observed by inverted microscope (Figure 1). The CTCs were grown in FBS enriched RPMI medium (10%) for the period of minimum 14 d on the membrane. The cultured cells were analyzed by means of histochemistry (May-Grünwald staining) and immunohistochemistry using the specific antibodies to identify cell origin [anti-cytokeratin 18 -FITC conjugated antibody (Sigma, Germany)], monoclonal CEA, cytokeratin CK7 and vimentin (Dako Denmark S/V) and unspecific DAPI staining (Sigma, Germany) (Figures 1, 2 and 3).

Alternatively the enriched CTCs fraction can be transferred from the membrane and cultured directly on any
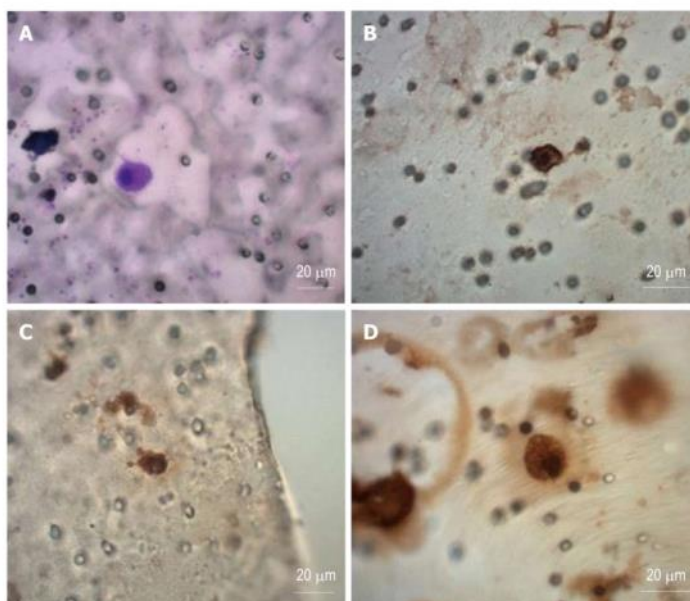
Figure 3 Circulating tumor cells captured on the separating membrane shown after different immunohistochemistry stainig proving the gastrointestinal origine of the captured cells. A: May-Gruenwald stain; B: CEA-antibody staining; C: CK7-antibody staining; D: Vimentin-antibody staining.

plastic surface or a microscopic slide. Microscopic slide culture is preferred if immunohistochemistry/immunofluorescence analysis is planned. If an intermediate CTCs-analysis is awaited, the CTCs-fraction is transferred in PBS (1.5 mL) to a cytospin slide. The slide is then dried for 24 h and analyzed by means of immunohistochemistry.

Cytomorphological analysis

The fixed and stained cells on the membrane were examined using light and fluorescent microscopy. The analytical process can be divided into two steps: (1) observing at smaller magnification (up to × 20) to identify cells or cell nuclei; and (2) observing at higher magnification (up to × 6) for more detailed evaluation of cytomorphology.

Cells captured on the separating membrane (single cells or cells within clusters) were located, digitized, and evaluated by a trained researcher and/or experienced pathologist. Cells presenting below listed characteristics were defined as CTCs: (1) nuclear size equal or larger than 10 μm); (2) presence of a visible cytoplasm; (3) prominent nucleoli; (4) high nuclear-cytoplasmic ratio, which is not necessarily true in case of *in vitro* cultured cells; and (5) irregular nuclear contour.

RESULTS

The frequency of the CTCs positivity is summarized for different patient subgroups in Figure 4. CTCs were detected in 16 patients of 24 patients (66.7 %), (Figure 4A), with comparable frequencies in operable and inop-

erable patients (60% *vs* 77.8%). Due to the low number of patients in the different disease stage groups, CTC-positivity does not reflect the disease stage (Figure 4B), tumor size (Figure 4C), or lymph node involvement (Figure 4D). The same percentage of CTC positivity was observed in the metastatic and nonmetastatic patients (66.7% *vs* 66.7%) (Figure 4E). Interestingly 88.9% patients with tumor grade 3 were affected by the spread of the pancreatic disease defined by CTCs (Figure 4F). The CTC - positivity reported for each patient individually is shown in the Table 2. We are not able to report any correlation of CTC-abundance and histology tumor subtype.

We evidence successful CTCs isolation in patients with pancreatic cancer, capturing viable cells with proliferation potential. The cells captured by size-based filtration approach are enriched with good fitness, what enables the culture of the CTCs unaffected by any antibodies or lysing solutions. The CTCs were cultured *in vitro* for further downstream applications. The confluent cell growth was reached in the majority of the cultivated CTC cases.

The size of the captured cells guided us in the cancer cell identification process even without any additional staining (*e.g.*, May-Grünwald -MGG). This standard staining protocol (MGG) has enabled us to analyze the nuclei including nucleoli. Generally the nucleus was bigger than 10 μm itself and the cells did not present much of cytoplasm immediately after separation process. The nuclear-cytoplasmatic ratio is relatively high in cancer cells, but not in the *in vitro* cultured CTCs. The CTCs
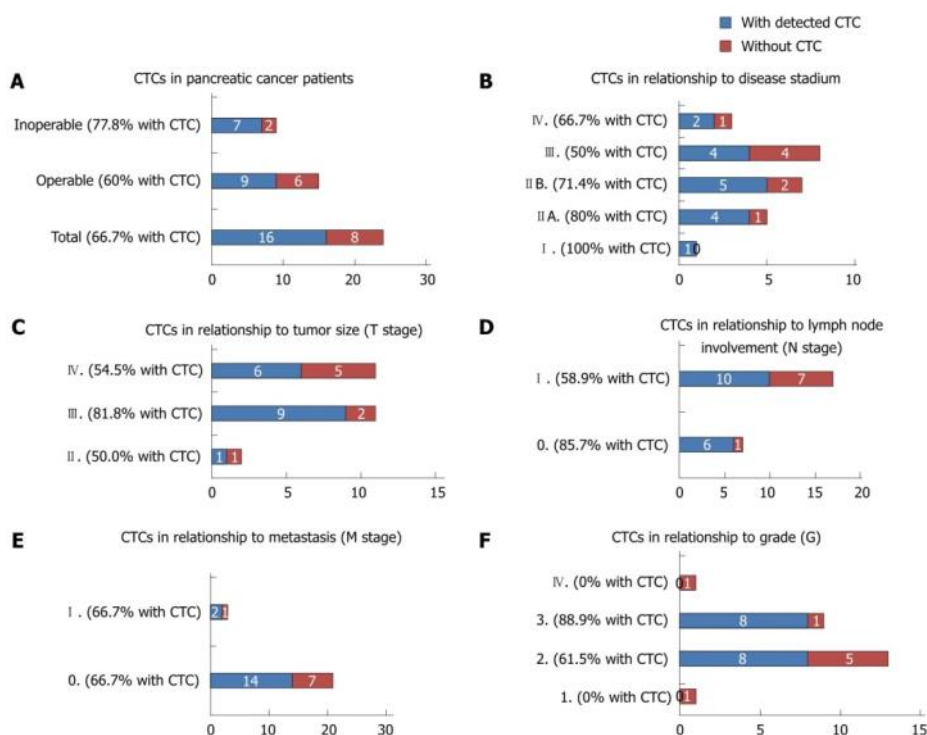
Figure 4 Circulating tumor cells positivity reported for patient subgroups. A: Circulating tumor cells (CTCs) in PaC patients, reflecting operability of the primary tumor; B: CTCs in PaC patients in different disease stages; C: CTCs in PaC patients, grouped according the tumor size; D: CTCs in PaC patients, grouped according the lymph node involvement; E: CTCs in PaC patients grouped according the metastasis; F: CTCs in PaC patients, grouped according to tumor grade.

get big and long in the culture, changing the nuclear-cytoplasmatic ratio. The cytoplasm of CTCs is rather pale than condensed.

Due to the cell size (15 μm), nucleus size (10 μm),shape, and nucleoli visualized by MGG or simple DAPI-stain in the formerly fixed cells, cancer cells are detected on the separating membrane (Figure1A), and also on the plastic bottom of the 6-well plate (Figure 1B-D). These results indicate that the captured cancer cells display plasticity enabling to grow through the separating membrane. Concerning the shape of the cancer cells in the "bottom fraction", these cells could present a more invasive CTC-fraction with a spindle cell-like shape. The immunohistochemical analysis has shown the abundance of the cytokeratin-18 in the "membrane" fraction as well as in the "bottom" fraction proving the carcinoma origin. We see an enormous potential in the gene expression analysis, which could reveal the epithelial - mesenchymal character of the detected cancer cells. The mesenchymal like type phenotype of CTCs after an epithelial- mesenchymal transition (EMT), was described by expression of CK7, CEA and vimentin (Figure 3).

DISCUSSION

The presented study aimed to successful isolation of CTCs from patients with PaC using a simple size-based separation device. The gentle antibody independent isolation process allows the subsequent CTC-cultivation *in vitro*.

The antibody independence could be of advantage because the detection of CTCs cannot be based only on the expression of epithelial makers (EpCAM) or cytokeratins due to their lost within EMT process[11-13].

The epithelial markers can be down regulated during tumor cell dissemination, affecting the detection rates of CTCs[14-16]. Relatively low CTC-detection rates were reported using the isolation methods relying on EpCAM expression (*e.g.*, CellSearch®, Adnagen®) in the early disease stages suggesting, EpCAM-based isolation processes may not be efficient for CTC-detection[17-19].

Two platforms for CTC- enumeration based on different isolation principles (CellSearch® and ISET) were compared in the study of pancreatic cancer by Khoja *et al*[20] prospectively. CellSearch® works on the antibody dependent principle of CTCs-separation. All CTCs expressing

Table 2 Individual patient characteristics and circulating tumor cells examination results

Stadium	Tumor histology	CTC	T	N	M	G	Surgery
I B	Ductal	YES	2	0	0	2	Operabile
II A	Cylindrocellular	YES	3	0	0	3	Operabile
II A	Ductal	YES	3	0	0	3	Operabile
II A	Ductal	YES	3	0	0	3	Operabile
II A	Ductal	NO	3	0	0	2	Inoperabile
II A	Ductal	YES	3	0	0	2	Operabile
II B	X	YES	3	1	0	3	Inoperabile
II B	Ductal	YES	3	1	0	3	Inoperabile
II B	X	NO	3	1	0	2	Inoperabile
II B	Ductal	YES	3	1	0	3	Operabile
II B	Ductal	YES	3	1	0	2	Operabile
II B	Ductal	YES	3	1	0	2	Inoperabile
II B	Anaplastic	NO	2	1	0	4	Operabile
III	Ductal	NO	4	1	0	2	Operabile
III	Ductal	NO	4	1	0	2	Operabile
III	Ductal/cylindrocellular	NO	4	1	0	1	Operabile
III	Ductal	YES	4	1	0	3	Inoperabile
III	Ductal/cribriform	NO	4	1	0	2	Operabile
III	Ductal	YES	4	0	0	2	Operabile
III	Ductal/neuroendocrine	YES	4	1	0	2	Inoperabile
III	Ductal	YES	4	1	0	3	Inoperabile
IV	Ductal	YES	4	1	1	2	Inoperabile
IV	Ductal	NO	4	1	1	3	Operabile
IV	Ductal	YES	4	1	1	2	Operabile

CTCs: Circulating tumor cells.

epithelial markers should be captured by immunomagnetic separation. ISET is a size-based, blood filtration device. It has been reported that CTCs were detected in 93% of patients *via* ISET and in 40 % by CellSearch®.

We detected CTCs in the 66.7% cases. If the patients are subcategorized, the CTC- positivity reaches 80% in some subgroups (*e.g.*, T3-stage). In discussing the results of the CTC-frequency in the pre-defined patient subgroups we should report the relationship between the CTCs and operability of the PaC patients. The CTCs were detected in the 77.8 % of inoperable patients. In the future the CTCs-tests could be used for pre-screening patients before operation. Stage III encompasses patients whose tumors can be surgically removed which represents more than a half of the patients. The CTCs positivity results show, that also in the resectable cases, the CTC-positivity number is very high (60%). There is a possibility to pre-treat the CTC-positive patients within neoadjuvant therapy (chemotherapy or radiotherapy) combined regimens. The survival analysis of the PaC patients in the tested group is not available yet to show how different the patient performance and therapy resulted following CTC testing.

We have to acknowledge the potential of CTCs in understanding and discovering the biological principles of cancer dissemination. The information on CTCs should help to manage patients according the CTC- presence. The *in vitro* proliferation of CTCs in PaC could enable an implementation of new analytical methodologies such as genome profiling, microRNA studies, protein expression

testing, chemoresistance analysis, and discovery of new therapeutic regimens and targets. To use the CTCs as biomarker monitoring the therapeutic efficiency in PaC could be of advantage in near future. Subsequently, CTC-testing is helping to improve patient stratification for new targeted drugs regimens[21-25].

Nevertheless, due to the lack of treatment possibilities in PaC, it is not possible yet to identify by help of CTCs new "druggable targets".But it is possible to identify in CTCs susceptible genes, which could be used for gene therapy development and immunotherapy optimization in individual patients[7].

CTCs may help to identify differences between primary tumor and metastasis on the phenotype and genotype level. The clonal heterogeneity of the primary tumor could explain differences between populations of disseminated cells and the evolution of their new genotypes and phenotypes[26]. To identify the differences on the gene expression level between the primary tumor cells and CTCs should be a base for improving the therapeutical approaches. To answer all the questions discussed above one needs larger number of CTCs. There are several ways how to obtain larger numbers of cells: (1) have more precise and specific separation techniques; (2) To analyze bigger blood volumes; or (3) culture CTCs *in vitro* enabling their proliferation and further downstream analysis. This could be reached by separation technologies as presented here. The newly reported successful *in vitro* CTCs-culture could be a tool to overcome the low-number CTCs limits, enabling any further research *e.g.*,

on protein expression level.

COMMENTS

Background
Metastasis in visceral tissue is the major cause of cancer death .The dissemination process is based on the plasticity of malignant cells enabling their migration from primary tumors to other microenvironments. Hematogenous dissemination is the most common dissemination route even in tumors spreading through lymphatic system. Circulating tumor cells (CTCs) are messengers in the cancer dissemination process-holding a promise to determine new therapeutical regimens for the advanced cancer. There have been many clinical studies which showed the utility of CTCs abundance in the bloodstream as a prediction and prognostic marker of the pancreatic disease.

Research frontiers
The authors still don't know yet how many tumor cells and how often are shed from the primary tumor into the bloodstream. It is hypothesized that 1 g of tumor tissue may release 10^6 of the cells into to bloodstream every 24 h. It is essential to establish sensitive and specific technologies to detect CTCs, which would enable their analysis on molecular level to further understand CTCs biology. CTCs present an opportunity to offer information on more effective treatment strategies and metastatic process prevention in individuals.

Innovations and breakthroughs
Innovation and improving of CTC detection methods has been reported recently for CTC microchips, filtration based methodologies, quantitative reverse transcription polymerase chain reaction. Improvements in CTC capture efficiency, quantification, imaging and molecular analyses are likely to enable further clinical applications. In this original research paper, we demonstrate that it is possible to isolate human CTCs from patients with pancreatic cancer, with subsequent cultivation and proliferation *in vitro*.

Applications
CTCs provide a novel prognostic and predictive biomarker enabling to monitor the efficacy of systemic therapy. CTCs may help to identify differences between primary tumor and metastasis on the phenotype and genotype level. The clonal heterogeneity of the primary tumor could explain differences between populations of disseminated cells and the evolution of their new genotypes and phenotypes. To identify the differences on the gene expression level between the primary tumor cells and CTCs, CTCs should be a base for improving the therapeutical approaches.

Terminology
CTCs the cancer cells circulating in the bloodstream. Size-based enrichment process: CTC-enrichment based on cell size, addresses the problem of reduced EpCAM expression.

Peer review
This paper provides a size-based method to isolate and culture pancreatic CTCs from clinical blood samples. The major point of this paper is the culture of CTCs. Many downstream tests are possible for the characterizations of these cells.

REFERENCES

1   **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10-29 [PMID: 22237781 DOI: 10.3322/caac.20138]

2   **De La Cruz MS**, Young AP, Ruffin MT. Diagnosis and management of pancreatic cancer. *Am Fam Physician* 2014; **89**: 626-632 [PMID: 24784121]

3   **Hackert T**, Büchler MW. Pancreatic cancer: advances in treatment, results and limitations. *Dig Dis* 2013; **31**: 51-56 [PMID: 23797123 DOI: 10.1159/000347178]

4   **Liberko M**, Kolostova K, Bobek V. Essentials of circulating tumor cells for clinical research and practice. *Crit Rev Oncol Hematol* 2013; **88**: 338-356 [PMID: 23830807]

5   **Eliasova P**, Kolostova K, Kobierzycki C, Bobek V. Clinical studies monitoring circulating and disseminated tumor cells in gastrointestinal cancers. *Folia Histochem Cytobiol* 2013; **51**: 265-277 [PMID: 24497131 DOI: 10.5603/FHC.2013.0037]

6   **Kaur S**, Baine MJ, Jain M, Sasson AR, Batra SK. Early diagnosis of pancreatic cancer: challenges and new developments. *Biomark Med* 2012; **6**: 597-612 [PMID: 23075238 DOI: 10.2217/bmm.12.69]

7   **Cen P**, Ni X, Yang J, Graham DY, Li M. Circulating tumor cells in the diagnosis and management of pancreatic cancer. *Biochim Biophys Acta* 2012; **1826**: 350-356 [PMID: 22683404 DOI: 10.1016/j.bbcan.2012.05.007]

8   **Mikulová V**, Kološtová K, Zima T. Methods for detection of circulating tumour cells and their clinical value in cancer patients. *Folia Biol* (Praha) 2011; **57**: 151-161 [PMID: 21978757]

9   **Ren C**, Han C, Zhang J, He P, Wang D, Wang B, Zhao P, Zhao X. Detection of apoptotic circulating tumor cells in advanced pancreatic cancer following 5-fluorouracil chemotherapy. *Cancer Biol Ther* 2011; **12**: 700-706 [PMID: 21811100 DOI: 10.4161/cbt.12.8.15960]

10   **Bobek V**, Kacprzak G, Rzechonek A, Kolostova K. Detection and cultivation of circulating tumor cells in malignant pleural mesothelioma. *Anticancer Res* 2014; **34**: 2565-2569 [PMID: 24778078]

11   **Raimondi C**, Gradilone A, Naso G, Vincenzi B, Petracca A, Nicolazzo C, Palazzo A, Saltarelli R, Spremberg F, Cortesi E, Gazzaniga P. Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Res Treat* 2011; **130**: 449-455 [PMID: 21298334 DOI: 10.1007/s10549-011-1373-x]

12   **Peeters DJ**, van Dam PJ, Van den Eynden GG, Rutten A, Wuyts H, Pouillon L, Peeters M, Pauwels P, Van Laere SJ, van Dam PA, Vermeulen PB, Dirix LY. Detection and prognostic significance of circulating tumour cells in patients with metastatic breast cancer according to immunohistochemical subtypes. *Br J Cancer* 2014; **110**: 375-383 [PMID: 24366294 DOI: 10.1038/bjc.2013.743]

13   **Fong D**, Moser P, Kasal A, Seeber A, Gastl G, Martowicz A, Wurm M, Mian C, Obrist P, Mazzoleni G, Spizzo G. Loss of membranous expression of the intracellular domain of EpCAM is a frequent event and predicts poor survival in patients with pancreatic cancer. *Histopathology* 2014; **64**: 683-692 [PMID: 24117877 DOI: 10.1111/his.12307]

14   **Lowes LE**, Allan AL. Recent advances in the molecular characterization of circulating tumor cells. *Cancers* (Basel) 2014; **6**: 595-624 [PMID: 24633084 DOI: 10.3390/cancers6010595]

15   **Joosse SA**, Pantel K. Biologic challenges in the detection of circulating tumor cells. *Cancer Res* 2013; **73**: 8-11 [PMID: 23271724 DOI: 10.1158/0008-5472.CAN-12-3422]

16   **Sterzynska K**, Kempisty B, Zawierucha P, Zabel M. Analysis of the specificity and selectivity of anti-EpCAM antibodies in breast cancer cell lines. *Folia Histochem Cytobiol* 2012; **50**: 534-541 [PMID: 23264216 DOI: 10.5603/17845]

17   **Farace F**, Massard C, Vimond N, Drusch F, Jacques N, Billiot F, Laplanche A, Chauchereau A, Lacroix L, Planchard D, Le Moulec S, André F, Fizazi K, Soria JC, Vielh P. A direct comparison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. *Br J Cancer* 2011; **105**: 847-853 [PMID: 21829190 DOI: 10.1038/bjc.2011.294]

18   **Hofman V**, Ilie MI, Long E, Selva E, Bonnetaud C, Molina T, Vénissac N, Mouroux J, Vielh P, Hofman P. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay™ and the isolation by size of epithelial tumor cell method. *Int J Cancer* 2011; **129**: 1651-1660 [PMID: 21128227 DOI: 10.1002/ijc.25819]

19   **Sandri MT**, Zorzino L, Cassatella MC, Bassi F, Luini A, Casadio C, Botteri E, Rotmensz N, Adamoli L, Nolè F. Changes in circulating tumor cell detection in patients with localized breast cancer before and after surgery. *Ann Surg Oncol* 2010; **17**: 1539-1545 [PMID: 20135356 DOI: 10.1245/s10434-010-0918-2]

20   **Khoja L**, Backen A, Sloane R, Menasce L, Ryder D, Krebs M, Board R, Clack G, Hughes A, Blackhall F, Valle JW, Dive C. A pilot study to explore circulating tumour cells in pan-

creatic cancer as a novel biomarker. *Br J Cancer* 2012; **106**: 508-516 [PMID: 22187035 DOI: 10.1038/bjc.2011.545]

21   **Gall TM**, Frampton AE, Krell J, Jacob J, Stebbing J, Jiao LR. Is the detection of circulating tumor cells in locally advanced pancreatic cancer a useful prognostic marker? *Expert Rev Mol Diagn* 2013; **13**: 793-796 [PMID: 24127751 DOI: 10.1586/14737159.2013.845091]

22   **Tjensvoll K**, Nordgård O, Smaaland R. Circulating tumor cells in pancreatic cancer patients: methods of detection and clinical implications. *Int J Cancer* 2014; **134**: 1-8 [PMID: 23447365 DOI: 10.1002/ijc.28134]

23   **Zhang L**, Riethdorf S, Wu G, Wang T, Yang K, Peng G, Liu J, Pantel K. Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. *Clin Cancer Res* 2012; **18**: 5701-5710

[PMID: 22908097 DOI: 10.1158/1078-0432.CCR-12-1587]

24   **Cristofanilli M**, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004; **351**: 781-791 [PMID: 15317891 DOI: 10.1056/NEJMoa040766]

25   **Lianidou ES**, Strati A, Markou A. Circulating tumor cells as promising novel biomarkers in solid cancers. *Crit Rev Clin Lab Sci* 2014; **51**: 160-171 [PMID: 24641350 DOI: 10.3109/10408363.2014.896316]

26   **Han L**, Chen W, Zhao Q. Prognostic value of circulating tumor cells in patients with pancreatic cancer: a meta-analysis. *Tumour Biol* 2014; **35**: 2473-2480 [PMID: 24218336 DOI: 10.1007/s13277-013-1327-5]

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REVIEW

Clinical studies monitoring circulating and disseminated tumor cells in gastrointestinal cancers

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Abstract: Circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) are responsible for the development of metastatic disease, and may also hold the key to determining tailored therapies of advanced cancer disease. Our review summarizes the prognostic significance of the detection of CTCs and DTCs in various gastrointestinal cancers with an overview of their possible use as prognostic biomarkers. This could be used in the future as a starting point for new clinical trials focusing on the predictive potential of circulating and disseminated tumor cells. (*Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 4, 265–277*)

Key words: circulating tumor cells; gastrointestinal cancer; esophageal cancer; colorectal cancer; gastric cancer; plastin3; prognosis

Abbreviations

AFP — alpha fetoprotein; BM — bone marrow; CD — cluster of differentiation; CEA — carcinoembryonic antigen; CHT — chemotherapy; CI — confidence interval; CTC — circulating tumor cell; CRC — colorectal carcinoma; CVB — central venous blood; CK — cytokeratin; DAPI — 4,6-diamidino-2-phenylindole; DFS — disease free survival; DTC — disseminated tumor cell; EpCAM — epithelial cell adhesion molecule; FISH — fluorescent *in situ* hybridization; 5-FU — 5-fluorouracil; HCC — hepatocellular carcinoma; HR — hazard ratio; ISET — isolation by size of epithelial tumor; ITC — isolated tumor cells; MACS — magnetic activated cell sorting; MFS — metastasis free survival; MSP — methylation specific polymerase chain reaction; MVB — mesenteric venous blood; NA — not available; OS — overall survival; PB — peripheral blood; PFS — progression-free survival; qPCR — quantitative real-time polymerase chain reaction; RFA — radiofrequency ablation; RT — radiotherapy; RT-PCR — reverse transcription polymerase chain reaction; TGFβ1 — transforming growth factor β1; TRC method — transcription reverse-transcription concerted method

Introduction

Single tumor cells occurring in blood circulation are called circulating tumor cells (CTCs), while the single tumor cells seeding distant organs prior to detection of metastasis are termed DTCs (disseminated tumor cells) [1]. CTCs and DTCs are believed to be responsible for the development of metastatic disease, as shown in the parallel-progression model of metastatic cascade [1, 2].

Over the last decade, various methods and systems have been developed to isolate and characterize CTCs and DTCs. The presence of these cells accompanies tumor invasion through the bloodstream and dissemination into other distant sites. Much effort has been necessary to understand the biology of cancer dissemination and to make clinical use of CTCs and DTCs. Our review summarizes the prognostic significance of

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the detection of circulating and disseminated tumor cells in various gastrointestinal cancers with a view of their future use in testing processes in clinical studies.

A cancer cell in circulation: a rare event

Recently, our understanding of cancer has considerably improved. While the basic definition of cancer remains unchanged, it is now considered a complex disease. CTCs and DTCs may be rare events of primary tumor progression. Many clinical studies have been conducted showing the utility of CTC detection in the peripheral blood as a valuable predictor of the clinical outcome for patients with solid tumors [3–5]. Detection, monitoring, and molecular analysis of these extremely rare cancer cells (estimated as one tumor cell per billion normal blood cells in patients with diagnosed metastatic cancer) could provide new possibilities in cancer treatment [6].

The methodology used for CTCs studies in gastrointestinal cancer has been reviewed in depth by Negin et al. [6]. There is no doubt that the development of new more sensitive detection techniques is crucial, and is aimed at gaining higher counts of CTCs and DTCs to make these methods into powerful tools of prediction. We have tried to produce a useful overview of recent methods of detection, isolation, and characterization of CTCs, such as immunomagnetic separation, flow cytometry, fluorescent *in situ* hybridization (FISH), and reverse transcription polymerase chain reaction (RT-PCR).

Nowadays, the only predictive marker used in colorectal carcinoma (CRC) is the *KRAS* gene, tested by gene mutational analysis. It is believed that we are close to discovering other genes for predictive purposes. This can also be achieved using CTCs, but their counts seem currently to be insufficient for proper analysis. CTC counts in analyzed peripheral blood in gastrointestinal cancers (*e.g.*, esophageal and gastric cancer), are low compared with other malignancies such as breast and prostate cancer. The absolute numbers in gastrointestinal cancers (such as metastatic colorectal cancer) are reported as 1–2 CTCs/7.5 mL of blood, while in metastatic prostate and breast cancer, counts are on the level of 3–5 and 6–7 CTCs/7.5 mL of blood, respectively [7–10]. It has been discussed that that liver could filter the blood coming in from the peritoneum, so CTCs may remain in the liver and occupy hepatic tissue, developing local metastasis [6]. This could be the reason that significantly higher rates of CTCs can be found analyzing mesenteric venous blood (MVB) in comparison to the peripheral blood [11]. This fact should be reflected in clinical studies, where perioperative blood sampling might be a source of CTCs for predictive analysis.

The range of possible diagnostic and therapeutic uses of CTCs is very wide. Firstly, monitoring cancer disease and demonstrating the therapeutic success achieved by molecular testing of CTCs (which in future may be known as 'liquid biopsy') are possible applications. Secondly, useful methods for inoperable patients where there is no other possibility of obtaining information about the tumor character (which could be called 'real-time tumor biopsy') is another option. In addition, it seems that CTCs and DTCs could provide a very good source of information about the chemosensitivity and chemoresistance of the primary tumor and about distant sites of metastasis [12].

However, very little is still known about the exact number of tumor cells released into the bloodstream by tumors in humans. It is hypothesized that 1 g of primary tumor may release 10^6 cells into the bloodstream every 24 hours [13]. It has been shown in orthotopic metastatic tumor animal models that surgical manipulation during oncological procedures may enhance the release of cancer cells from the primary tumor site into the circulation. Pressure, biopsy, and laser treatments can all dramatically increase CTC counts (up to sixty-fold), whereas proper tumor resection significantly decreases CTC count [14]. Similarly, increases in CTC counts have been show in human clinical studies of radiofrequency ablation (RFA) — a method of tissue destruction that uses the heat generated by high-frequency alternating current. CTCs from patients with CRC liver metastases were quantified prior to and immediately after open surgery, laparoscopic resection, and open or percutaneous RFA. Surgical procedures led to a statistically significant decrease in CTC counts measured at multiple sites (peripheral vein and artery, hepatic portal vein, hepatic vein). Conversely, RFA, whether open or percutaneous, was associated with a significant increase in CTC count [15]. It may be expected that *in vivo* detection of intervention-amplified CTCs could be used in the future for early diagnosis of small tumors undetectable with conventional methods [14].

Clinical impact of CTCs in clinical studies in patients with esophageal, gastric, and colorectal cancer

The clinical relevance of CTC analysis in gastrointestinal cancers is summarized in Tables 1–4, in which studies are listed according to the diagnosis and the CTC detection method. Table 1 presents immunocytological analysis, whereas Table 2 shows RT-PCR analysis of CTCs in esophageal, gastric, and pancreatic cancers. Similarly, in Tables 3 and 4, immunocytological and RT-PCR based studies of CTCs in colorectal carcinoma are presented. Some of the more interesting results are discussed below.

Table 1. CTCs/DTCs in esophageal and gastric cancer — immunocytological studies

Clinical Study	Year	Pa-tients (n)	Stage	Sampling	CTC	DTC	Diagnostic method	OS (months)	P-value (OS)	Note
Vashist et al. [18]	2012	362	Mets, non-mets	Pre- and post-operative	–	Yes	ICC, CK assay	DTCs– OS 39,9 DTCs– DFS 28,2 DTCs+ OS 13,6 DTCs+ DFS 9,7	P < 0.001	Esophageal cancer, DTCs in bone marrow identified by CK (cytokeratin), detailed clinico-pathologic patient characteristic
Matsu-saka et al. [28]	2010	52	Mets, non-mets	At baseline, during therapy	Yes	–	Cell-Search®	2 week point CTCs– OS 3,5 CTCs– PFS 4,9 CTCs+ OS 11,7 CTCs+ PFS 1,4 4 week point CTCs– OS 4,0 CTCs– PFS 5,0 CTCs+ OS 11,4 CTCs+ PFS 1,4	P ≤ 0.001	Gastric cancer, detailed clinico-pathologic patient characteristic
Hiraiwa et al. [7]	2008	171	Mets, non-mets	Pre- and post-operative	Yes	–	Cell-Search®	NA	P = 0.343 (EC) P = 0.032 (GC)	Esophageal cancer (EC), gastric cancer (GC)
Kolo-dziejczyk et al. [44]	2007	32	Mets, non-mets	Before and after preoperative CHT	Yes	Yes	IF	CTCs– 22,6 CTCs+ 20,3	P = 0.683	Gastric cancer effects of pre-operative CHT on CTCs/DTCs

CHT — chemotherapy; CK — cytokeratin; CTC — circulating tumor cell; DFS — disease-free survival; DTC — disseminated tumor cell; EC — esophageal cancer; GC — gastric cancer; ICC — immunocytochemistry; IF — immunofluorescence; Mets — metastases; NA — not available; OS — overall survival; PFS — progression-free survival

Table 2. CTCs/DTCs in esophageal and gastric cancers — gene expression based studies

Clinical Study	Year	Pa-tients (n)	Stage	Sampling	CTC	DTC	Dia-gnostic method	Mole-cular markers	OS (mon-ths)	P-value (OS)	Note
Yin et al. [19]	2012	72	Mets, non-mets	Pre- and post-radiotherapy	Yes	–	RT-PCR	CK19, CEA, survivin	–	NA	CTC (+) post-radiothe-rapy prognostic factor for ESCC apart from patients' Karnofsky performance status scores.
de Albuqu-erque et al. [45]	2012	247	Non-mets	Pre- and post-operative	Yes	–	RT-PCR	KRT19, MUC1, EPCAM, CE-ACAM5, BIRCS, SCGB2A2, ERBB2	NA	NA	CTC (+) 66.7% in esophageal, 62.2% in gastric, 33.3% in small intestine, 60.6% in co-lon, and 66.7% in rectal adenocarcinomas
Hoff-mann et al. [46]	2009	59	NA	Pre- and post-operative	Yes	–	Density gradient, RT-PCR	Methy-lated DAPK or APC promoter	poor	P = 0.04	–

→

Table 2. cd.

Braben-der et al. [47]	2008	29	Non-mets	Prior neoadjuvant CHT	Yes	–	Density gradient, RT-PCR	ERCC1	NA	NA	ERCC1 mRNA expression associated with response to neoadjuvant RT
Hoff-mann et al. [48]	2007	62	NA	Pre- and post-operative	Yes	–	Density gradient, RT-PCR	Survivn	NA	P < 0.04	Survivin mRNA levels fall after surgical resection
Liu et al. [17]	2007	53	Non-mets	Pre- and post-operative, 3rd post-operative day	Yes	–	Density gradient, RT-PCR	CEA	NA	P < 0.05	Patients with high levels CEA in CTC fraction showed mets 1 year after surgery more often
Ikoma et al. [49]	2007	44	Mets, non-mets	Preoperative	Yes	–	RT-PCR, MSP	p16, E-cadherin, RARbeta	NA	P = 0.05	Methylation--specific PCR (MSP)
Hoff-mann et al. [50]	2007	44	NA	Postoperative	Yes	–	Density gradient (On-coQuick) RT-PCR	Survivin	NA	P < 0.04	Gastric, esophageal CRC, pancreatic: survivin levels fall after complete surgical resection
Ikeguchi et al. [51]	2005	59	NA	Pre- and post-operative	Yes	–	RT-PCR	CEA	NA	P = 0.064	Gastric cancer
Ito et al. [52]	2004	28	NA	NA	Yes	–	RT-PCR	CEA, CK20	NA	NA	–
Kaganoi et al. [53]	2004	70	NA	Pre-, intra- and post-operative	Yes	–	RT-PCR	SCCA	NA	P < 0.001	Squamous cell carcinoma Antigen (SCCA)
Huang et al. [54]	2003	62	NA	Preoperative	Yes	–	RT-PCR	CEA, CK19, CK20	NA	NA	Gastrointestinal cancer
Nakashi-ma et al. [55]	2003	54	NA	Preoperative	Yes	–	RT-PCR	CEA	NA	NA	–
Koike et al. [56]	2002	33	Mets, non-mets	Pre-, intra- and post-operative, 1 week after surgery	Yes	–	RT-PCR	Del-taNp63	NA	NA	–
Miyazo-no et al. [57]	2001	57	Mets, non-mets	Pre- and post-operative	Yes	–	RT-PCR	CEA	NA	NA	Gastric cancer, surgical manipu-lation
Soeth et al. [58]	1997	245	Mets, non-mets	Preoperative	Yes, 104	Yes, 141	RT-PCR	CK20 for DTCs	CK20 RNA+ shorter OS	P > 0.0001 (CRC) P = 0.0414 (GC), NA (PC)	Gastric cancer (GC), colorectal cancer (CRC), pancreatic cancer (PC)

CHT, CK, CTC, DFS, DTC, GC, Mets, NA, OS – same as described for Table 1. BIRCs — BIR-containing proteins; CEA — carcinoembryonic antigen; CEACAM5 — carcinoembryonic antigen-related cell adhesion molecule 5; CRC — colorectal cancer; DAPK — death-associated protein kinase; ESCC — esophageal squamous cell carcinoma; EpCAM — epithelial cell adhesion molecule; ERBB2 — erythroblastic leukemia viral on-cogene homolog 2; ERCC1 — excision repair cross-complementing 1 protein; MSP — methylation specific polymerase chain reaction; MUC1 — mucin 1; PC — pancreatic cancer; RT-PCR — reverse transcription polymerase chain reaction; SCGB2A2 — secretoglobin family 2A member 2.

Table 3. CTCs/DTCs in colorectal cancer — immunocytological studies

Clinical Study	Year	Patients (N)	Stage	Sampling	CTC	DTC	Diagnostic method	OS (months)	P-value (OS)	Note
Galletti et al. [59]	2013	Art. in press	Art. in press	Art. in press	Yes	–	Immunomagnetic (antiEpCAM Ab)	Art. in press	Art. in press	Gastric cancer
Sato et al. [60]	2012	42	Mets, non-mets	NA	Yes	–	CellSearch® TRC method	NA	$P = 0.0494$ (CellSearch) $P = 0.0317$ (TRC)	A correlation between CTC detection and prognosis for both methods in 25 patients
Rahbari et al. [10]	2012	200 (CVB) 80 (MVB)	Mets, non-mets	Intraoperative	Yes	–	CellSearch®	NA	NA	Detailed clinicopathologic patient characteristic; CTCs in the central venous blood (CVB) in comparison with mesenteric venous blood (MVB), plus CEA, CA19-9 in serum
Aggarwal et al. [61]	2012	430	Mets	At baseline, during therapy	Yes	–	CellSearch®	CTCs– (CEA < 25 ng/mL) 19.9 (CEA ≥ 25 ng/mL) 20.8 (CEA < 50 ng/mL) 22.5 (CEA ≥ 50 ng/mL) 17.5 CTCs+ (CEA ≥ 25 ng/mL) 11.7 (CEA ≥ 50 ng/mL) 12.1	$P = 0.001$	Detailed clinicopathologic patient characteristic; correlation of CTCs and CEA
Sastre et al. [32]	2012	180	Mets	At baseline, after therapy	Yes	–	CellSearch®	after therapy CTCs– OS 25,1 CTCs– PFS 12 CTCs+ OS 17,7 CTCs+ PFS 7,8	$P = 0.0059$ (at baseline) $P = 0.0095$ (after therapy)	Bevacizumab vs. bevacizumab+CHT (TTD MACRO study)
Flatmark et al. [62]	2011	235	Mets, non-mets	Intraoperative	–	Yes	Immunomagnetic (antiEpCAM Ab), ICC (pan-CK Ab)	EpCAM+	$P = 0.006$ (EpCAM+) $P = 0.06$ (CK+)	EpCAM, CK immunomagnetic in comparison with ICC
Matsusaka et al. [63]	2011	64	Mets	At baseline, after therapy	Yes	–	CellSearch®	CTCs– OS 29,1 CTCs– PFS 10,4 CTC+ OS 10,2 CTCs+ PFS 7,3	$P < 0.001$	CTCs independently predict PFS and OS before and during CHT
Tol et al. [64]	2010	451	Mets	At baseline, during therapy	Yes	–	CellSearch®	at baseline CTCs– OS 22 CTCs– PFS 10,5 CTCs+ OS 13,7 CTCs+ PFS 8,1	$P < 0.0001$	CTCs independently predict PFS and OS before and during CHT (CAIRO2 Trial)

Table 3. cd.

	Year	N	Mets/non-mets	Timing			Method		P	
Papavasiliou et al. [65]	2010	20	Mets, non-mets	Pre-, intra- and post-operative	Yes	Yes	ICC, CellSearch®	NA	P = 0.036	Correlation between the presence of postoperative CTCs and OS, DFS, CTC detection increases during liver intraoperative manipulation
Königsberg et al. [66]	2010	38	Mets	At baseline, during therapy	Yes	–	ICC	NA	P = 0.007 (PFS)	PFS significant difference between mCRC patients without detectable CTCs and with > 1 CTCs
Marrinuci et al. [67]	2010	5	Mets	During therapy	Yes	–	IF	NA	NA	Time since diagn., location of mets, therapy line, intrapatient pleomorphism (primary/metastatic colon tumor cells vs. CTCs)
Maestro et al. [68]	2009	195	Mets, non-mets	Postoperative, before systemic therapy	Yes	–	CellSearch®	NA	P < 0.001	More than 2 CTCs/7.5 mL is a frequent event in mets. cases
Wong et al. [69]	2009	132	Mets, non-mets	Preoperative, postoperative before systemic therapy	Yes	–	Immunomagnetic detection	NA	P < 0.001	CK20 early recurrence
Cohen et al. [70]	2008	430	Mets	At baseline, during therapy	Yes	–	CellSearch®	at baseline CTCs– OS 18,5 CTCs– PFS 7,9 CTCs+ OS 9,4 CTCs+ PFS 4,5	P < 0.0001	CTCs independent predictor of PFS and OS in pre/post 1.,2.,3.- line of CHT
Hiraiwa et al. [7]	2008	171	Mets, non-mets	Pre- and post-operative	Yes	–	CellSearch®	NA	P = 0.005	OS for patients with > 2 CTCs was shorter
Schopp-meyer et al. [71]	2006	47	Mets	Pre- and post-operative	Yes	Yes	ICC, CellSearch®	NA	NA	DTCs does not predict extrahepatic recurrence in patients undergoing surgery for hepatic metastases
Cohen et al. [31]	2006	49	Mets	At baseline, during therapy	Yes	–	ICC	NA	P = 0.001	Elevated count of CTCs for patients with tumor progression vs. nonprogression

CHT, CK, CTC, DTC, Mets, NA, OS, PFS – same as described for Tables 1 and 2. Ab — antibody; CVB — central venous blood; EpCAM — epithelial cell adhesion molecule; ICC — immunocytochemistry; IF — immunofluorescence; MVB — mesenteric venous blood; TRC method — transcription reverse-transcription concerted method

Table 4. CTCs and DTCs in colorectal cancer — gene-expression based studies

Clinical Study	Year	Patients (N)	Stage	Sampling	CTC	DTC	Diagnostic method	Molecular markers	OS (months)	P-value (OS)	Note
Lu et al. [41]	2013	90	Non-mets Stage III	Postoperative, before and after CHT	Yes	–	RT-PCR	CEA, CK19, CK20	NA	Significantly shorter OS/DFS	Post-CHT CTCs+ is a potential powerful surrogate marker (FOLFOX after curative resection) CTC better relapse predictor than CEA serum levels
Barbazán et al. [72]	2012	14	Mets	NA	Yes	–	EpCAM-based immuno-separation qRT-PCR	TGFβ1, TIMP1, CLU	NA	P < 0.0001	TGFβ1,TIMP1,CLU - linked to aggressive phenotype (poor prognosis)
Iinuma et al. [73]	2011	735	Mets Dukes stage A/B/C	Preoperative	Yes	–	RT-PCR	CEA, CK19,CK20, CD133	NA	P < 0.001	Poor prognosis, high recurrence risk
Gazzaniga et al. [43]	2010	40	Mets	During treatment	Yes	–	EpCAM-based immuno-separation, RT-PCR	ALDH, survivin, MRP5	2–9 months (PFS)	P < 0.046 (ALDH1) P < 0.001 (Survivin, MRP5)	In CTCs+ and ALDH1, survivin, MRP5 = significantly shorter PFS
Vogelaar et al. [74]	2010	46	Mets	Preoperative	–	Yes	ICC, RT-PCR	CK20	NA	P = 0.002	Patients with RT-PCR negative bone marrow had a significantly better OS
Yen et al. [75]	2009	76	Mets	Unspecified	Yes	–	RT-PCR	KRAS	NA	P < 0.0001	Wild-type KRAS are more likely to have a better PFS and OS when treated with cetuximab + CHT
Koyanagi et al. [76]	2008	34	Mets, non-mets	Preoperative	Yes	–	qPCR	C-MET, MAGE-A3, GalNAc-T, CK20	NA	P = 0.045	All tested markers have an independent prognostic value for OS
Uen et al. [40]	2008	438	Mets, non-mets	Postoperative	Yes	–	RT-PCR	hTERT, CK19 CK20/CEA	NA	P < 0.001 (RFS)	Poor RFS
Zieglschmid et al. [77]	2007	76	Mets, non-mets	Pre- and post-operative	–	Yes	immunomagnetic selection RT-PCR	CEA, EGFR,-GA733	NA	NA	EGFR / CEA gene expression correlated with disease progression and tumor stage CEA expression in DTCs has a relapse predictive value
Allen-Mersh et al. [78]	2007	196	NA	Pre- and post-operative	Yes	–	RT-PCR	CEA, CK20	NA	NA	CEA/CK20+ within 24h of primary CRC resection is a strong predictor of CRC recurrence
Wang et al. [79]	2007	157	Mets, non-mets	Pre- and post-operative	Yes	–	RT-PCR	CEA, hTERT, CK19, CK20	Poor	P < 0.05	Poor RFS

Table 4. cd.

Sadahiro et al. [80]	2007	200	NA		Yes	–	RT-PCR	CEA, CK, CD133	Poor	P = 0.007 (DFS) P = 0.04 (OS)	Poor DFS
Iinuma et al. [81]	2006	128	NA	Pre- and post-operative	Yes	–	RT-PCR	CEA/CK20	NA	NA	CEA/CK20 mRNA in tumor drainage blood has prognostic value
Koch et al. [82]	2005	37	Mets	Pre-, intra- and post-operative	Yes	Yes	RT-PCR	CK20	NA	P = 0.009 (CTCs) P = 0.013 (DTCs)	CTCs found itraoperatively are independent prognostic factor
Staritz et al. [83]	2004	42	Mets	Before and during palliative CHT	Yes	–	RT-PCR	CK20	CTCs+ 53 weeks CTCs– 86 weeks	NA	Shorter OS in CTCs+
Vlems et al. [84]	2003	41	Mets	Preoperative	Yes	Yes	RT-PCR	CK20	NA	NA	DTCs did not predict subsequent extrahepatic recurrence
Ito et al. [85]	2002	99	NA	Pre- and post-operative	Yes	–	RT-PCR	CEA, CK, CD133	NA	P = 0.03	Poor DFS
Patel et al. [86]	2002	116	Mets, non-mets	Pre- and post-operative	Yes	–	RT-PCR	CEA, CK20	NA	NA	Correlation of CEA/CK20 to Duke stage
Wyld et al. [87]	1998	25	Mets	Min. 3 months after CHT	Yes	–	RT-PCR	CK20	NA	NA	Correlation of CK 20 in the peripheral blood and DFS/OS

CEA, CHT, CK, CRC, CTC, DFS, DTC, GC, Mets, NA, OS — same as described for Tables 1, 2 and 3. ALDH1 — aldehyde dehydrogenase 1; CD — cluster of differentiation; CLU — clusterin; EGFR — epidermal growth factor receptor; EpCAM — epithelial cell adhesion molecule; hTERT — human telomerase reverse transcriptase; MAGE-A3 — melanoma-associated antigen 3; MRP5 — Multidrug resistance-associated protein 5; qPCR — quantitative real-time polymerase chain reaction; RFS — relapse-free survival; RT-PCR — reverse transcription polymerase chain reaction; TIMP — tissue inhibitor of metalloproteinase; TGFβ1 — transforming growth factor β1

Esophageal cancer (EC)

Esophageal cancer (EC) is notorious for its aggressive biological behavior, local infiltration, involvement of adjacent lymph nodes, and broad metastasis through hematogenous spread. It has been reported that the frequency of hematogenous recurrence is high, despite radical surgery with lymph node dissection [16]. In this regard, the detection of cancer cells in the blood could be important for identifying patients with a high risk of relapse. There have been many studies showing a positive correlation between detection of CTCs, tumor staging, and patient prognosis. Detection of CTCs from PB of EC patients by conventional qPCR methods has been reported for several genes.

Liu et al. [17] aimed at establishing a quantitative system for evaluating the role of CTCs in PB from patients who underwent surgical resection during esophageal cancer treatment. 155 PB samples from 53 EC patients were collected before surgery (B-1), immediately after surgery (B0), and on the third postoperative day (B+3). A direct qPCR method based on CEA mRNA gene expression was designed for the detection of CTCs. The authors showed significant differences between groups B-1 *vs.* B0 ($p = 0.0001$) and B-1 *vs.* B+3 ($p = 0.0209$). 50% of the patients with $R > 0.4$ (R = CTC ratio of B+3 over B0) showed tumor recurrence within 1 year after surgery, whereas the probability was only 14.3% for patients with $R < 0.4$ ($p = 0.043$). The prognostic utility of CTCs in EC has been shown also in studies where the gene expression of survivin, ERCC1, and APC has been tested by RT-PCR, as shown in Table 2.

The prognostic relevance of the presence of DTCs in bone marrow (BM) for the postoperative course of EC has also been evaluated recently [18]: 370 patients with EC diagnosis (189 squamous cell carcinomas and 181 adenocarcinomas), were surgically treated with complete resection (R0). They received neither adjuvant nor neoadjuvant therapy. DTCs were detected by an immunocytochemical cytokeratin assay in preoperatively taken BM aspirates. Overall, 120 (32.4%) patients harbored DTCs in their BM. The presence of DTCs significantly correlated with aggressive tumor biology, as indicated by increased tumor size ($p = 0.026$), regional ($p = 0.002$) and distant ($p = 0.012$) lymph node metastases, and higher relapse rate ($p < 0.001$, χ^2 test). The presence of DTCs in bone marrow was a very strong and independent prognostic factor in patients with resectable EC [18].

The CTC status in the PB of patients with esophageal squamous cell carcinoma (ESCC), before and after radiotherapy (RT), was evaluated by Yin et al. [19]. A total of 72 ESCC patients enrolled in this study were treated with radical RT. The nested RT-PCR reaction was used to detect the three representative markers of CTCs: CEA, CK19, and survivin. The results showed that the presence of CTCs, and the positive expression of at least one of these three markers in patients with ESCC pre-RT and post-RT were 54.2% and 38.9%, respectively ($p = 0.059$). Furthermore, the analysis of the patients according to lymph node metastasis and adverse 2-year progression-free survival (PFS) revealed changes in CTC status after RT, which would reflect patients' response to RT. In a multivariate analysis with the Cox proportional hazard model, only CTC positivity post-RT was an independent, unfavorable prognostic factor for ESCC, apart from subsequent chemotherapy and patients' Karnofsky performance status scores (a scale quantifying cancer patients' general well-being). In conclusion, the positive detection of CTCs in patients with ESCC after RT may be a promising biomarker for radiation efficiency and prognosis assessment in ESCC [19].

Gastric cancer (GC)

Follow-up studies on gastric cancer (GC) patients suggested that CTC-positive cases with increased burdens of CTCs were associated with poorer prognoses than CTC-negative cases. The situation was similar with DTCs [15]. Both localized and metastatic GC can shed detectable concentrations of CTCs into the blood. The presence of CTCs in circulation suggests not only a high risk of tumor recurrence, but also an unfavorable clinical outcome even in the early stages of GC [20]. The prognostic impact of CTCs in GC has been reported in several studies [21–27]. The sensitivity of RT-PCR CTC detection was superior to the other less commonly used cytological detection methods involving fluorescence-activated cell sorting (FACS), immunohistochemistry (IHC), and immunocytochemistry (ICC) [20]. For the identification of CTCs in GC, different markers and their combinations were tested in the analyzed studies. The combination of EpCAM, CK8, CK18, and CK19 seems to be prognostically the most relevant in GC [7, 24]. On the other side, single survivin expression also achieved prognostic significance in at least 2 studies [29, 30]. Based on the analyzed data, detection of CTCs might be used as a noninvasive method, not only for the confirmation of GC diagnosis, but also for estimation of prognosis.

Colorectal cancer (CRC)

In general, the detection of CTCs in colorectal cancer (CRC), independently of the method and markers

used, correlates with the stage of the cancer disease [31, 32]. On the other side, the correlation of CTCs with some known clinicopathological prognostic factors (*e.g.*, T4 tumor size, perineural invasion, bowel obstruction, high preoperative CEA levels) is still uncertain [32]. It is believed that the correlation of CTCs with clinicopathological factors would increase if the sensitivity of the CTC detection were higher. CTC positivity is observed in approximately 40–50% of metastatic CRC patients. Differences in CTCs detection can be observed depending on the sampling site, as shown by Rahbari et al. [10], who tested compartmental differences of CTC in CRC. The qualitative and quantitative detection of CTCs was higher in the mesenteric venous blood (MVB) than in the central venous blood (CVB) of patients with CRC. It has been speculated that the liver works as a filter and stops CTCs from entering the central circulation [6]. Moreover, higher counts of CTCs were detected when the tumor was localized in the lower part of rectum than in the cases of middle and high rectal involvement [6, 33].

The biomarkers used for the CTCs detection in cytological or RT-PCR examination of patients with CRC are listed in Tables 3 and 4. Generally, the EpCAM pre-enrichment is a basis for further cytokeratine (CK19/20, CK8/18) and CEA testing. Recently, plastin3 has been shown to have significant clinical relevance. Plastin3 positivity in the PB was found to be associated with clinicopathological risk factors, such as depth of invasion, lymph node and liver metastasis, presence of peritoneal dissemination, increased recurrence rate, and higher Dukes stage. It is very important to note that plastin3 expression was also detected in all patients with recurrent disease, and at a level higher than in the case of prerecurrence and of patients without recurrence [34]. The correlation between CTCs and prognosis in CRCs was stronger if CKs and multiple markers were used than for the one-marker assay [35].

Recently, several meta-analyses evaluating the prognostic value of CTC examination in CRC have been published. Rahbari et al. [36] included 36 studies and 3094 patients in their final meta-analysis. The pooled analyses combining all sampling sites (PB, mesenteric PB (MPB), and BM) associated the detection of CTCs/DTCs with poor recurrence-free survival (RFS). Stratification by sampling site showed that detection of CTCs in the PB compartment was a statistically significant prognostic factor, but that detection in the MPB or BM was not.

Similarly, 12 studies representing 1329 patients were suitable for pooled analysis of CRC patients in a prognostic study [37]. The OS and PFS were worse in CTC-positive patients, whereas analyzing PFS separately, the subgroup with significantly worse survival rate contained over 35% CTC-positive patients. Multivariate analysis was performed on eight studies and identified the detection of CTCs as an independent prognostic factor for survival. Moreover, the meta-analysis reported that the detection of CTCs in PB of patients with resectable colorectal liver metastases, or with widespread metastatic CRC, was associated with disease progression and poor survival [37]. The study of Katsuno et al. [38] highlights the potential importance of cancer cell detection in the venous drainage of colorectal cancers as a prognostic marker and a mode of staging in this neoplastic disease.

Regarding the effect of chemotherapy on CTC counts, it has been evaluated that the prognosis of patients with undetectable CTCs after chemotherapy was significantly better [39]. Additionally, molecular detection of persistent postoperative CTCs has been confirmed as a prognostic marker of early relapse in I–III stage CRC patients, which could help to select patients for an enhanced follow-up and therapeutic program [40, 41].

In summary, it is expected that CTCs and DTCs will be used for mutational analysis of the genes connected directly to the targeted therapy (*e.g.*, *KRAS*, *BRAF*). The heterogeneity of the genetic profiles of cells from the primary tumor, metastatic tumors, and CTCs may be an explanation for the variable response to EGFR-inhibitor chemotherapy [5, 42, 43]. CTCs are not only a marker for advanced disease, but also have prognostic and predictive potential. A decrease in CTC levels during chemotherapy is correlated with improved responses to chemotherapy [39].

Several very important questions need to be answered, and further studies are required to unify the isolation techniques before CTCs can be adapted for widespread clinical use. In particular, the following questions should be central to future research: Can CTCs be used to define a group of patients with "resectable" metastases who should not undergo resection? Can CTCs be used to monitor the immediate effectiveness of systemic chemotherapy or to predict which chemotherapy would be most effective? Can CTCs be used to help staging patients with metastatic CRCs?

Finally, we are reminded that stage IV of colorectal cancer is a disease with many possible outcomes, ranging from rapid death to recovery [40]. We also recall that our ability to predict which patient will experience which outcome is relatively limited. The detection of CTCs is a potentially promising biomarker that could contribute to the staging of the cancer and this deserves a prospective study.

Conclusion

In summary, it is essential to establish sensitive, specific technologies to detect CTCs. More detailed analyses of their molecular characteristics should be performed with the aim of understanding the biology of CTCs and DTCs. This may provide a yet-untapped option to develop therapeutic strategies that will effectively treat and prevent metastatic process for each person individually.

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References

1. Steinert G, Schölch S, Koch M et al. Biology and significance of circulating and disseminated tumor cells in colorectal cancer. *Langenbecks Arch Surg.* 2012;397:535–542.
2. Brechot C et al. Molecular analysis of circulating tumor cells isolated by ISET: A diagnostic approach to improve follow up and treatment of patients with cancer. Boston, World CTC Summit, 2012.
3. Takeuchi H, Kitagawa Y. Detection of circulating tumor cells in gastrointestinal cancer: Has its time come? *J Gastrointest Oncol.* 2012;3:84–85.
4. Maheswaran S, Haber DA. Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev.* 2010;20:96–99.
5. Kin C, Kidess E, Poultsides GA et al. Colorectal cancer diagnostics: biomarkers, cell-free DNA, circulating tumor cells and defining heterogeneous populations by single-cell analysis. *Expert Rev Mol Diagn.* 2013;13:581–599.
6. Negin BP, Cohen SJ. Circulating tumor cells in colorectal cancer: past, present, and future challenges. *Curr Treat Options Oncol.* 2010;11:1–13.
7. Hiraiwa K, Takeuchi H, Hasegawa H et al. Clinical significance of circulating tumor cells in blood from patients with gastrointestinal cancers. *Ann Surg Oncol.* 2008;15:39092–39100.
8. Moreno JG, O'Hara SM, Gross S et al. Changes in circulating carcinoma cells in patients with metastatic prostate cancer correlate with disease status. *Urology.* 2001;58:386–392.
9. Dong X, Alpaugh KR, Cristofanilli M. Circulating tumor cells (CTCs) in breast cancer: a diagnostic tool for prognosis and molecular analysis. *Chin J Cancer Res.* 2012;24:388–398.
10. Rahbari NN, Bork U, Kircher A et al. Compartmental Differences of Circulating Tumor Cells in Colorectal Cancer. *Ann Surg Oncol.* 2012;19:2195–2202.
11. Iwanicki-Caron I, Basile P, Toure E et al. Usefulness of Circulating Tumor Cell Detection in Pancreatic Adenocarcinoma Diagnosis. *Am J Gastroenterol.* 2013;108:152–155.
12. Juratli MA, Sarimollaoglu M, Siegel E et al. Real-time monitoring of circulating tumor-cell release during tumor manipulation using in vivo photoacoustic and fluorescent flow cytometry. *Head Neck.* 2013; DOI: 10.1002/hed.23439. [Epub ahead of print].
13. Butler TP, Gullino PM. Quantitation of cell shedding into efferent blood of mammary adenocarcinoma. *Cancer Res.* 1975;35:512–516.
14. Jiao LR, Apostolopoulos C, Jacob J et al. Unique localization of circulating tumor cells in patient with hepatic metastases. *J Clin Oncol.* 2009;36:6160–6165.
15. Wang GY, Li Y, Yu YM et al. Detection of disseminated tumor cells in bone marrow of gastric cancer using magnetic activated cell sorting and fluorescent activated cell sorting. *J Gastroenterol Hepatol.* 2009;24:299–306.
16. Katayama A, Mafune K, Tanaka Y et al. Autopsy findings in patients after curative esophagectomy for esophageal carcinoma. *J Am Coll Surg.* 2003;196:866–873.
17. Liu Z, Jiang M, Zhao J et al. Circulating tumor cells in perioperative esophageal cancer patients: quantitative assay system and potential clinical utility. *Clin Cancer Res.* 2007;13:2992–2997.
18. Vashist YK, Effenberger KE, Vettorazzi E et al. Disseminated tumor cells in bone marow and the natural course of resected esophageal cancer. *Ann Surg.* 2012;255:1105–1112.
19. Yin XD, Yuan X, Xue JJ et al. Clinical significance of carcinoembryonic antigen-, cytokeratin 19-, or survivin-positive circulating tumor cells in the peripheral blood of esophageal squamous cell carcinoma patients treated with radiotherapy. *Dis Esophagus.* 2012;25:750–756.
20. Zhang ZY, Ge HY. Micrometastasis in gastric cancer. *Cancer Lett.* 2013;336:34–45.
21. Arigami T, Uenosono Y, Hirata M et al. B7–H3 expression in gastric cancer: a novel molecular blood marker for detecting circulating tumor cells. *Cancer Sci.* 2011;102:1019–1024.
22. Saad AA, Awed NM, Abd Elkerim NN et al. Prognostic significance of E-cadherin expression and peripheral blood micrometastasis in gastric carcinoma patients. *Ann Surg Oncol.* 2010;17:3059–3067.
23. Pituch-Noworolska A, Kolodziejczyk P, Kulig J et al. Circulating tumour cells and survival of patients with gastric cancer. *Anticancer Res.* 2007;27:635–640.
24. Yeh KH, Chen YC, Yeh SH et al. Detection of circulating cancer cells by nested reverse transcription-polymerase chain reaction of cytokeratin-19 (K19)-possible clinical significance in advanced gastric cancer. *Anticancer Res.* 1998;18:1283–1286.
25. Koga T, Tokunaga E, Sumiyoshi Y et al. Detection of circulating gastric cancer cells in peripheral blood using real time quantitative RT-PCR. *Hepatogastroenterology.* 2008;84:1131–1135.
26. Illert B, Fein M, Otto C et al. Disseminated tumor cells in the blood of patients with gastric cancer are an independent predictive marker of poor prognosis. *Scand J Gastroenterol.* 2005;40:843–849.
27. Uen YH, Lin SR, Wu CH et al. Clinical significance of MUC1 and c-Met RT-PCR detection of circulating tumor cells in patients with gastric carcinoma. *Clin Chim Acta.* 2006;367:55–61.
28. Matsusaka S, Chin K, Ogura M et al. Circulating tumor cells as a surrogate marker for determining response to chemotherapy in patients with advanced gastric cancer. *Cancer Sci.* 2010;101:1067–1071.
29. Bertazza L, Mocellin S, Marchet A et al. Survivin gene levels in the peripheral blood of patients with gastric cancer independently predict survival. *J Transl Med.* 2009;7:111.
30. Cao W, Yang W, Li H et al. Using detection of survivin-expressing circulating tumor cells in peripheral blood to predict tumor recurrence following curative resection of gastric cancer. *J Surg Oncol.* 2011;103:110–115.
31. Cohen SJ, Alpaugh RK, Gross S et al. Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. *Clin Colorectal Cancer.* 2006;6:125–132.

32. Sastre J, Maestro ML, Gómez-Espa a A et al. Circulating tumor cell count is a prognostic factor in metastatic colorectal cancer patients receiving first-line chemotherapy plus bevacizumab: a Spanish Cooperative Group for the Treatment of Digestive Tumors study. *Oncologist.* 2012;17:947–955

33. Molnar B, Ladanyi A, Tanko L et al. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. *Clin Cancer Res.* 2001;12:4080–4085.

34. Yokobori T, Iinuma H, Shimamura T et al. Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. *Cancer Res.* 2013;73:2059–2069.

35. Torino F, Bonmassar E, Bonmassar L et al. Circulating tumor cells in colorectal cancer patients. *Cancer Treat Rev.* 2013;39:759–772.

36. Rahbari NN, Aigner M, Thorlund K et al. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. *Gastroenterology.* 2010;138:1714–1726.

37. Groot Koerkamp B, Rahbari NN, Büchler MW et al. Circulating tumor cells and prognosis of patients with resectable colorectal liver metastase or widespread metastatic colorectal cancer: a meta-analysis. *Ann Surg Oncol.* 2013;20:2156–2165.

38. Katsuno H, Zacharakis E, Aziz O et al. Does the presence of circulating tumor cells in the venous drainage of curative colorectal cancer resections determine prognosis? A meta-analysis. *Ann Surg Oncol.* 2008;15:3083–3091.

39. Neki K, Kawahara H, Watanabe K et al. Usefulness of circulating tumor cells after preliminary chemotherapy for prediction of response to further anticancer therapy in patients with initially unresectable metastatic colorectal cancer. *Anticancer Res.* 2013;33:1769–1772.

40. Uen YH, Lu CY, Tsai HL et al. Persistent presence of postoperative circulating tumor cells is a poor prognostic factor for patients with stage I–III colorectal cancer after curative resection. *Ann Surg Oncol.* 2008;15:2120–2128.

41. Lu CY, Uen YH, Tsai HL et al. Molecular detection of persistent postoperative circulating tumour cells in stages II and III colon cancer patients via multiple blood sampling: prognostic significance of detection for early relapse. *Br J Cancer.* 2011;104:1178–1184.

42. Lankiewicz S, Zimmermann S, Hollmann C et al. Circulating tumour cells as a predictive factor for response to systemic chemotherapy in patients with advanced colorectal cancer. *Mol Oncol.* 2008;3:349–355.

43. Gazzaniga P, Gradilone A, Petracca A et al. Molecular markers in circulating tumour cells from metastatic colorectal cancer patients. *J Cell Mol Med.* 2010;14:2073–2077.

44. Kolodziejczyk P, Pituch-Noworolska A, Drabik G et al. The effects of preoperative chemotherapy on isolated tumor cells in the blood and bone marrow of gastric cancer patients. *Br J Cancer.* 2007;97:589–592.

45. de Albuquerque A, Kubisch I, Ernst D et al. Development of a molecular multimarker assay for the analysis of circulating tumor cells in adenocarcinoma patients. *Clin Lab.* 2012;58:373–384.

46. Hoffmann AC, Vallböhmer D, Prenzel K et al. Methylated DAPK and APC promoter DNA detection in peripheral blood is significantly associated with apparent residual tumor and outcome. *J Cancer Res Clin Oncol.* 2009;135:1231–1237.

47. Brabender J, Vallböhmer D, Grimminger P et al. ERCC1 RNA expression in peripheral blood predicts minor histopathological response to neoadjuvant radio-chemotherapy in patients with locally advanced cancer of the esophagus. *J Gastrointest Surg.* 2008;12:1815–1821.

48. Hoffmann AC, Warnecke-Eberz U, Luebke T et al. Survivin mRNA in peripheral blood is frequently detected and significantly decreased following resection of gastrointestinal cancers. *J Surg Oncol.* 2007;95:51–54.

49. Ikoma D, Ichikawa D, Ueda Y et al. Circulating tumor cells and aberrant methylation as tumor markers in patients with esophageal cancer. *Anticancer Res.* 2007;27:535–539.

50. Hoffmann AC, Warnecke-Eberz U, Luebke T et al. Survivin mRNA in peripheral blood is frequently detected and significantly decreased following resection of gastrointestinal cancers. *J Surg Oncol.* 2007;95:51–54.

51. Ikeguchi M, Kaibara N. Detection of circulating cancer cells after a gastrectomy for gastric cancer. *Surg Today.* 2005;35:436–441.

52. Ito H, Kanda T, Nishimaki T et al. Detection and quantification of circulating tumor cells in patients with esophageal cancer by real-time polymerase chain reaction. *J Exp Clin Cancer Res.* 2004;23:455–464.

53. Kaganoi J, Shimada Y, Kano M et al. Detection of circulating oesophageal squamous cancer cells in peripheral blood and its impact on prognosis. *Br J Surg.* 2004;91:1055–1060.

54. Huang P, Wang J, Guo Y et al. Molecular detection of disseminated tumor cells in the peripheral blood in patients with gastrointestinal cancer. *J Cancer Res Clin Oncol.* 2003;129:192–198.

55. Nakashima S, Natsugoe S, Matsumoto M et al. Clinical significance of circulating tumor cells in blood by molecular detection and tumor markers in esophageal cancer. *Surgery.* 2003;133:162–169.

56. Koike M, Hibi K, Kasai Y et al. Molecular detection of circulating esophageal squamous cell cancer cells in the peripheral blood. *Clin Cancer Res.* 2002;8:2879–2882.

57. Miyazono F, Natsugoe S, Takao S et al. Surgical maneuvers enhance molecular detection of circulating tumor cells during gastric cancer surgery. *Ann Surg.* 2001;233:189–194.

58. Soeth E, Vogel I, Röder C et al. Comparative analysis of bone marrow and venous blood isolated from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. *Cancer Res.* 1997;57:3106–3110.

59. Galletti G, Sung MS, Vahdat LT et al. Isolation of breast cancer and gastric cancer circulating tumor cells by use of an anti HER2-based microfluidic device. *Lab Chip.* 2014;14:147–156.

60. Sato N, Hayashi N, Imamura Y et al. Usefulness of Transcription-Reverse Transcription Concerted Reaction Method for Detecting Circulating Tumor Cells in Patients With Colorectal Cancer. *Ann Surg Oncol.* 2012;19:2060–2065.

61. Aggarwal C, Meropol NJ, Punt CJ et al. Relationship among circulating tumor cells, CEA and overall survival in patients with metastatic colorectal cancer. *Ann Oncol.* 2013;24:420–428.

62. Flatmark K, Borgen E, Nesland JM et al. Disseminated tumour cells as a prognostic biomarker in colorectal cancer. *Br J Cancer.* 2011;104:1434–1439.

63. Matsusaka S, Suenaga M, Mishima Y et al. Circulating endothelial cells predict for response to bevacizumab-based chemotherapy in metastatic colorectal cancer. *Cancer Chemother Pharmacol.* 2011;68:763–768.

64. Tol J, Koopman M, Miller MC et al. Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. *Ann Oncol.* 2010;5:1006–1012.

65. Papavasiliou P, Fisher T, Kuhn J et al. Circulating tumor cells in patients undergoing surgery for hepatic metastases from colorectal cancer. *Baylor Univ Med Cent Proc.* 2010;23:11–14.

66. Königsberg R, Gneist M, Jahn-Kuch D et al. Circulating tumor cells in metastatic colorectal cancer: efficacy and feasibility of different enrichment methods. *Cancer Lett.* 2010;293:117–123.

67. Marrinucci D, Bethel K, Lazar D et al. Cytomorphology of Circulating Colorectal Tumor Cells: A Small Case Series. *J Oncol.* 2010;2010:861341.

68. Maestro LM, Sastre J, Rafael SB et al. Circulating Tumor Cells in Solid Tumor in Metastatic and Localized Stages. *Anticancer Res.* 2009;29:4839–4843.

69. Wong SC, Chan CM, Ma BB et al. Clinical significance of cytokeratin 20-positive circulating tumor cells detected by a refined immunomagnetic enrichment assay in colorectal cancer patients. *Clin Cancer Res.* 2009;15:1005–1012.

70. Cohen SJ, Punt CJ, Iannotti N et al. Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients With Metastatic Colorectal Cancer. *J Clin Oncol.* 2008;26:3213–3221.

71. Schoppmeyer K, Frühauf N, Oldhafer K et al. Tumor cell dissemination in colon cancer does not predict extrahepatic recurrence in patients undergoing surgery for hepatic metastases. *Oncol Rep.* 2006;15:449–454.

72. Barbazán J, Alonso-Alconada L, Muinelo-Romay L et al. Molecular Characterization of Circulating Tumor Cells in Human Metastatic Colorectal Cancer. *PLoS One.* 2012;7:e40476.

73. Iinuma H, Watanabe T, Mimori K et al. Clinical significance of circulating tumor cells, including cancer stem-like cells, in peripheral blood for recurrence and prognosis in patients with Dukes' stage B and C colorectal cancer. *J Clin Oncol.* 2011;29:1547–1555.

74. Vogelaar FJ, Mesker WE, Rijken AM et al. Clinical impact of different detection methods for disseminated tumor cells in bone marrow of patients undergoing surgical resection of colorectal liver metastases: a prospective follow-up study. *BMC Cancer.* 2010;10:153.

75. Yen LC, Yeh YS, Chen CW et al. Detection of KRAS oncogene in peripheral blood as a predictor of the response to cetuximab plus chemotherapy in patients with metastatic colorectal cancer. *Clin Cancer Res.* 2009;15:4508–4513.

76. Koyanagi K, Bilchik AJ, Saha S et al. Prognostic Relevance of occult nodal micrometastases and circulating tumor cells in colorectal cancer in a prospective multicenter trial. *Cli Cancer Res.* 2008;22:7391–7396.

77. Zieglschmid V, Hollmann C, Mannel J et al. Tumor-associated gene expression in disseminated tumor cells correlates with disease progression and tumor stage in colorectal cancer. *Anticancer Res.* 2007;27:1823–1832.

78. Allen-Mersh TG, McCullough TK, Patel H et al. Role of circulating tumour cells in predicting recurrence after excision of primary colorectal carcinoma. *Br J Surg.* 2007;94:96–105.

79. Wang JY, Lin SR, Wu DC et al. Multiple molecular markers as predictors of colorectal cancer in patients with normal perioperative serum carcinoembryonic antigen levels. *Clin Canc Res.* 2007;13:2406–2413.

80. Sadahiro S, Suzuki T, Maeda Y et al. Detection of carcinoembryonic antigen messenger RNA-expressing cells in peripheral blood 7 days after curative surgery is a novel prognostic factor in colorectal cancer. *Ann Surg Oncol.* 2007;14:1092–1098.

81. Iinuma H, Okinaga K, Egami H et al. Usefulness and clinical significance of quantitative real-time RT-PCR to detect isolated tumor cells in the peripheral blood and tumor drainage blood of patients with colorectal cancer. *Int J Oncol.* 2006;28:297–306.

82. Koch M, Kienle P, Hinz U et al. Detection of hematogenous tumor cell dissemination predicts tumor relapse in patients undergoing surgical resection of colorectal liver metastases. *Ann Surg.* 2005;241:199–205.

83. Staritz P, Kienle P, Koch M et al. Detection of disseminated tumour cells as a potential surrogate-marker for monitoring palliative chemotherapy in colorectal cancer patients. *J Exp Clin Cancer Res.* 2004;23:633–639.

84. Vlems FA, Diepstra JH, Punt CJ et al. Detection of disseminated tumour cells in blood and bone marrow samples of patients undergoing hepatic resection for metastasis of colorectal cancer. *Br J Surg.* 2003;90:989–995.

85. Ito S, Nakanishi H, Hirai T et al. Quantitative detection of CEA expressing free tumor cells in the peripheral blood of colorectal cancer patients during surgery with real-time RT-PCR on a LightCycler. *Cancer Lett.* 2002;183:195–203.

86. Patel H, Le Marer N, Wharton RQ et al. Clearance of circulating tumor cells after excision of primary colorectal cancer. *Ann Surg.* 2002;235:226–231.

87. Wyld DK, Selby P, Perren TJ et al. Detection of colorectal cancer cells in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. *Int J Cancer.* 1998;79:288–293.

8.2 Publikace *in extenso*, které jsou podkladem disertace bez IF

ELIASOVA P., KOLOSTOVA K., JAKABOVA A., PINKAS M., BOBEK V., GÜRLICH R.; Circulating tumor cells in tumors of gastrointestinal tract-personalized therapy. Selected papers from European Federation Congress of the International College of Surgeons in Praque and Pilsen,2015; EurSurg (25 August 2016);48(3): S179-S226. ISSN: 1682-8631 (Print) 1682-4016 (Online). DOI: 10.1007/s10353-016-0442-0

the left retroperitoneum after failure of initial surgery and four cycles of chemotherapy (histologically closed as a high-grade sarcoma type poorly differenciated liposarcoma with component of rhabdomyosarcoma with heterologous components created by dedifferentiation of pre-existing low-grade liposarcoma). The course on ERAS was in all pts without complications with normal healing and pts were discharged home on common diet with renewed bowel movement, fully mobilized in pts after RP + EPLND release 8 and 7 days after the operation, in the case of RC + EPLND after 18 and 16 days after surgery retroperitoneal sarcoma after 8 days. Also, another course was without complications, with no signs of disease recurrence at a mean of 6 months. Pts after RP + EPLND well restored voiding stereotype and sufficient 1–2 pads/day.

Discussion: As a counterpart to SS another modalities have been developed for salvage local treatment of radioreccurent tumors. When comparing for example long-term results of salvage cryotherapy and salvage high-intensity focused ultrasound with salvage RP for radioreccurent PCa there was demonstrated superiority and better cancer control rate for SS. Of all the local salvage treatment options that salvage RP currently offers pts the greatest likelihood of a cure. Salvage RP is also not associated with so high financial demands as the others mentioned modality, which at the time of scarce financial resources is also not negligible. Salvage RP is currently considered the standard of local salvage therapy for radioreccurent PCa. Generally SS is a surgically challenging but effective secondary local treatment of radiorecurrent tumours with curative intent. The surgeon's experience plays an important role in the outcome of demanding surgical techniques, especially in terms of SS and perioperative treatment-related complications. The frequency of complications has decreased in modern series of RP. Functional recovery, as in tolerance of food without nausea and regained mobility, was considered the most important target of recovery. Interdisciplinary ERAS approach to control perioperative and postoperative pathophysiology and rehabilitation and its principles help us reduced complications and LOS like for other major surgery. In the context of previous studies that have confirmed the benefits of early rehabilitation and pelvic floor muscle training we continue with this interdisciplinary approach also after discharge from surgery. Early biofeedback-pelvic floor muscle training not only hastens the recovery of urinary continence after radical prostatectomy but allows for significant improvements in the severity of incontinence, voiding symptoms and pelvic floor muscle strength 12 months postoperatively. Among other possibilities of individualization of ERAS comes to better preparing for SS (smoking cessation and reduction of alcohol intake 4 weeks prior to surgery; encouraging regular physical exercise etc.).

Conclusions: Early recovery after ERAS for SS for pts with radioreccuren malignant tumors appears to have significant benefits in terms of postoperative morbidity, quality of life, and reduction of LOS, similar as for primary surgery.

Circulating tumor cells in tumors of gastrointestinal tract – personalized therapy

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Background: Circulating tumor cells (CTCs) are responsible for the development of metastatic disease, and may also hold the key to determining tailored therapies of advanced cancer disease.

Methods: Detection of CTCs were based on the combination of the cytomorphological characterisation and gene expression analysis which are repeated with an interval (the time of operation, prior adjuvant therapy after the end of therapy).

Results: Both, the evaluation of CTCs/DTCs presence and analysis of chemoresistance genes expression in CTCs/DTCs were correlated with clinical status of disease (localization tumor, TNM classification, stage, grade, extent surgery).

Conclusion: We presented some of patients with tumor in gastrointestinal tract in the form of short case study.

Keywords: gastrointestinal cancer, circulating tumor cells, biomarker

Abbreviations: CA – carbohydrate antigen, CD – lymphocyte common antigen, CEA – carcinoembryonic antigen, cell-free DNA – cell-free deoxyribonucleic acid, CK – cytokeratin, CTC – circulating tumor cell, CRC – colorectal carcinoma, DTC – disseminated tumor cell, EDTA – ethylenediaminetetraacetic acid, EGFR – epidermal growth factor receptor, EUS – endoscopic ultrasonography, ERCC1 – excision Repair Cross-Complementation Group 1 gene, EpCAM – epithelial cell adhesion molecule, FU – 5-fluorouracil, GIST – gastrointestinal stromal tumor, KRT18 – cytokeratin 18, MDR – multi-drug resistance gene, microRNA – micro ribonucleic acid, MRI – magnetic resonance imaging, MRP – multidrug resistance-associated protein, MUC1 – mucin1, PB – peripheral blood, qPCR – quantitative real-time polymerase chain reaction, RRM1,2 – ribonucleotide reductase M1,2, TIMP-1 – tissue inhibitor of metalloproteinases 1

Introduction: Over the last decade, we have seen some interesting data emerging that has essentially changed the treatment of patients with gastrointestinal cancer. Several important strides have been made in the management of metastatic colorectal cancer including the introduction of biomarkers and the recognition that anti-EGFR should only be given to patients whose tumors are RAS wild type.

At present, the only predictive marker used in colorectal carcinoma (CRC) is the K-RAS gene which was tested by gene mutational analysis. It is believed that we are close to discovering other genes for predictive purposes.

Although numerous significant technological and methodological advances, gastrointestinal oncology research has not yielded novel blood biomarkers suitable for population-wide screening purposes. The presently available (CEA, CA 19-9, CA 125) and developing (cell-free DNA, microRNA, single protein marker TIMP-1, CTCs) biomarkers are used.

At our surgery department we try to use CTCs as a predictive and prognostic biomarker and its other clinical applications for patients with tumors of gastrointestinal tract (GIT) to help surgeon and other clinicians make treatment decisions (good timing of surgery and follow up), predict future metastasis, and monitor disease recurrence, respectively. Subsequently, according to the last developments indicate a possible reoperation.

The circulating tumor cells (CTCs) are single tumor cells occurring in blood circulation, while the single tumor cells seeding distant organs prior to detection of metastasis are termed DTCs (disseminated tumor cells). Recently, our understanding of cancer has significantly improved. While the basic definition of cancer remains unchanged, it is now considered a complex disease. CTCs and DTCs may be rare events of primary tumor progression. Many clinical studies have been conducted showing the utility of CTCs detection in the peripheral blood as a valuable predictor of the clinical outcome for patients with solid tumors.

The range of possible diagnostic and therapeutic uses of CTCs is very wide. Firstly, determination and monitoring cancer disease achieved by molecular testing of CTCs (which in future may be known as 'liquid biopsy') are possible applications. For example in patients with pancreatic tumor there are negative biomarkers available, ambiguous or negative findings by CT/MRI imaginations, negative biopsy by EUS, and tumor is the verified up during surgery – often already inoperable. Integration of CTC into available oncomarkers allows improved diagnosis and gives the opportunity to indicate patients for surgery at an earlier stage of the disease.

Secondly, useful methods for inoperable patients where there is no other possibility of obtaining information about the tumor character (which could be called 'real-time tumor biopsy') are another option. In addition, it seems that CTCs and DTCs could provide a very good source of information about the chemosensitivity and chemoresistance of the primary tumor and about distant sites of metastasis.

Our aim is to integrate testing into CTC routine care of patients with oncosurgery, similarly, as for surgical patients with sepsis complications and chronic defects are identified infectious agents and used targeted antimicrobial therapy according to its sensitivity.

Detection, monitoring, and molecular analysis of circulating tumor cells could provide new possibilities in cancer treatment. This may provide a yet-untapped option to develop therapeutic strategies (neoadjuvant, adjuvant and palliative oncological treatment with specifying the appropriate timing surgery), that will effectively treat and prevent metastatic process for each person individually, specifying the appropriate timing surgery, that will effectively treat and prevent metastatic process for each person individually.

Materials and Methods: For each patient, peripheral blood (8 mL) was withdrawn into test tubes containing 1.6 mg EDTA/mL blood as an anticoagulant. In selected patients was withdrawn venous blood from tumor, peritoneal lavage and tumor tissue within surgery, minimally one of them. Peripheral blood (PB) samples was collected perioperative and postoperative. A new size-based separation MetaCell® technology (Metacell s. r. o.,Ostrava,Czech republic) was used for enrichment and cultivation of CTCs *in vitro*. The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (pores with 8 μm diameter). The membrane filter is kept in a plastic ring that is transferred into the 6-well cultivation plate; 2 mL RPMI media is added to the filter top and 2 mL to the well bottom.

CTCs are cultured on the membrane *in vitro* under standard cell culture conditions (37 °C, 5 % CO_2). The cultured cells have been analyzed by confocal imaging using fluorescent stains by means of cytomorphology criteria and were further analysed by gene expression analysis of tumor specific (cytokeratin 7, cytokeratin 18, cytokeratin 19, cytokeratin 20, EpCAM, MUC1, EGFR, chromogranin) and white blood cells markers (CD45 a CD68). In the occurrence of CTCs positivity, the molecular analysis was supplemented by determination of chemoresistance genes expression (ERCC1, MDR1, MRP1, 2, 4, 5, and 7, RRM1, 2) and analysis of mutations in K-RAS gene were performed.

Case study: 68-years old man was examined for blood in stool and rectal tenses. In January of 2015 underwent colonoscopy with finding adenocarcinoma stage G2 with recto sigmoid stenosis at 20 cm from dental line. Preoperatively was assessed as a stage II (T4NXM0). Anterior resection of the recto sigma was performed. There were palpable two resistance in liver (2 cm and 5 mm average) – suspected metastasis. Sigmoideorectoanastomosis end-to-end was constructed by circular stapler 33 mm. Resection of propagate by stenosis circular tumor length of 8 cm was assessed R0.
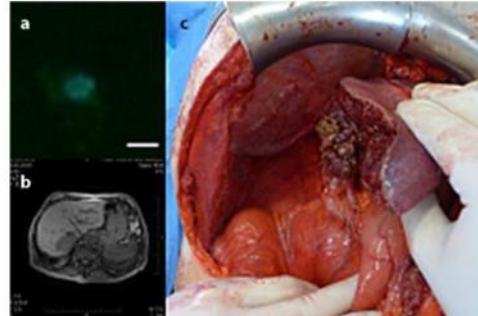

Fig. 1 a) Circulating tumor cells isolated from peripheral blood within CRC-surgery, cultured in vitro. The cells out of the in vitro celture have been analyzed by time-lapse corfocal imaging using fluorescent stains (viable cells are stained by nonspecific nuclear fluorescent stain (NucBlueTM). Bar represents 10μm. The cells were further analyzed by gene expresion in comparision, b) metastasis liver of S7 imaging by MRI, c) metastasis liver of S7 imaging by perioperative photo

		T	TS				T	TS
1	ACTB	X			14	MRP1	X	
2	CD45	X			15	MRP2	X	
3	CD68	X			16	MRP3	X	
4	EPCAM	X	X		17	MRP4	X	
5	MUC1	X			18	MRP5	X	X
6	TTF1				19	MRP7	X	X
7	KRT5				20	MDR1		
8	KRT6				21	ERCC1	X	X
9	KRT7	X	X		22	RRM1	X	X
10	KRT18	X			23			
11	KRT19				24	HPRT	X	
12	KRT20	X						
13	EGFR	X						

Table 1 Differentlai diagnostic markers tested by qPCR (T-Gene expression analysis tumor assodated genes (KRT7, KRT18, KRT19, KRT20, EpCAM, MUCl, EGFR, CHGA), white blood cell markers (CD45 ,C068) and chemoresistance genes (ERCCl, MDRl, MRP1,2,4,5,7)

. MRP1, MRP2 marker of resistance to anthracyclines
· MRP2, **MRP5** markers of resistance to cisplatin and carboplatin
· MRP2, **MRP7** markers of resistance to taxanes
· MRP1, MRP2, MRP4 markers of resistance to irinotecan and topotecan
· MRP1, MRP2 markers of resistance for cyclophosphamide and other alkylants
· **MRP5** markers for 5-FU resistance
· MRP1, **MRP7** markers of vinca alkaloids resistance
· MRP2, MRP4, **MRP5** markers for methotrexate resistence,gemcitabine rezistance
.ERCC1 – cisplatina, karboplatina

.RRM1, RRM2 - gemcitabine

Table 2 The chemorezistance-associated genes