Original Article
Circulating tumor cells: what we know, what do we want to know about them and are they ready to be used in clinics?

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Abstract: Circulating tumor cells (CTC) present in peripheral blood are assigned precursors of advanced tumor disease. Simplicity of blood withdrawal procedure adds practically an unlimited possibility of the CTC-monitoring and the advantages of the repeated biopsies over time. CTC got prognostic, predictive and diagnostic status with the technologic advance. Although the clinical utility of CTC has reached the high evidence, the significance of CTC testing was presented in the treatment strategy mostly with palliative intention. We report on the experiences with the CTC-testing in the CLIA-like laboratory working with the size-based CTC separation and *in vitro* culture. The data is presented in the form of case reports in patients with breast (BC), colorectal (CRC), prostate (PC) and lung cancer (NSCLC) to support the clinical utility of CTC during the neoadjuvant, adjuvant and palliative treatment. The presented findings support the evidence for liquid biopsy clinical implementation and enhance the ability of malignant disease monitoring and the treatment efficacy prediction.

Keywords: Circulating tumor cells, breast cancer, colorectal cancer, non-small-cell lung cancer, prostate cancer, chemoresistance

Introduction

The count of CTC in blood of oncological patients is very low [1]. Detection of CTC is also limited by their heterogeneity. Finally, the capacity of various malignancies to release CTC into the peripheral circulation is different depending on the stage of the disease and also on the type of malignancy [2-12]. The chance of CTC positivity is in general notably higher in metastatic than in the primary disease, e.g. 2-55% in primary BC vs. 40-80% in metastatic BC [13].

Technologies try to overcome the rare occurrence of CTC by using the enrichment step to separate CTC from blood cells. The negative selection is based on the elimination of leukocyte fraction (e.g. by using anti-CD45 antibod-

ies) from blood. The positive selection utilizes surface features of CTC or their physical properties such as cells size or density. The most commonly used methods are based on the immunomagnetic selection of CTC with epithelial features. But these methods are impoverished to detect CTC with the lack of epithelial characteristics, e.g. cancer cells whose phenotype has been altered by the process of epithelial-mesenchymal transition (EMT) [14] or which have the character of stem cells [15]. Combinations of different methods and new approaches [16], in particular microfluidic systems, focus on increasing the sensitivity and specificity of CTC selection and detection.

The standardization of technologies at all levels of CTC identification and results interpretation based on different approaches is still a prob-

Table 1. Clinical indications to CTC examination

Prediction of disease response to neoadjuvant chemotherapy
Indication of "additional" adjuvant therapy in residual disease
Observation after adjuvant therapy
CTC monitoring after adjuvant therapy and during metastatic disease
CTC-testing after resection of metastases and early prediction of disease relapse
Assessment of KRAS mutation status from CTC
Strategy of using CTC for the palliative treatment guidance
Typing of tumors with unknown primary site or duplicate tumors

lem. The rare occurrence of CTC in non-metastatic disease is the reason why the threshold for the CTC positivity is different in the primary disease in comparison to the metastatic malignancy. Therefore the prognostic significance of CTC demonstrated by the use of various approaches has not the same weight.

The only FDA (U.S. Food and Drug Administration) approved method for the detection of CTC (Cellsearch®) is based on the separation of EpCAM (epithelial cell adhesion molecule) positive cells. Published data declare the prognostic significance of CTC detected by Cell search in metastatic BC [17], metastatic CRC and PC [2, 3].

The size-based enrichment protocol of CTC reported in our study enables capturing and *in vitro* cultivation of viable CTC (MetaCell®). CTC can be further analysed by the downstream molecular analysis (e.g. gene expression testing by qPCR). Previous studies indicated a fast and simple enrichment in various cancer types [4, 18-20].

In general, the prognostic significance of CTC is supported by several studies on the level of primary disease: in primary BC pre- and postoperatively [21, 22], in early CRC preoperatively [23], from the postoperative lavage of the peritoneal cavity [24] and after the adjuvant chemotherapy [25], or in early NSCLC [26].

The clinical utility of CTC is still the subject of clinical studies [27-30]. Well known SWOG s0500 trial did not support the assumption of the clinical benefit of early chemotherapy change in patients with the metastatic BC and persistent CTC after the first cycle of therapy [31]. The characterisation of CTC was not performed in this study so the "real" predictive significance of CTC was not considered. Conversely, the use of anti-HER2 antibody ther-

apy in patients with metastatic BC and HER2-positive (HER2+) CTC resulted in the prolongation of the time to progression compared to the patients who were not treated with the targeted therapy [32]. CTC detection and characterisation in patients with metastatic castrate-resistant PC (CRPC) can select a group of patients who will most probably not benefit from the hormonal therapy [33]. Splice variant 7 of the androgen receptor (AR-V7) detected in CTC predicts tumor response to the hormonal therapy and taxanes. The patients with AR-V7 positive CTC treated with taxanes survived longer than those treated with the hormonal therapy [34].

The assumption is that the characteristics of CTC are more important than the total CTC number. Below presented individual case reports describe our experience with CTC examination in patients with BC, CRC, PC and NSCLC and the potential for their use in the clinical practice. Every case report is documented in the appropriate figure. The scheme of the disease course, patients' characteristics and results of CTC-testing are shown in pictures schematically. Questions asked by clinicians that lead to the indication of CTC-testing, are in relation to several therapy relevant points (**Table 1**). CTC examination was indicated only as an additional test to a standard diagnostics.

Material and methods

Before the blood collection, patients were informed about the purpose and nature of the examination and their agreement with testing was reaffirmed by the signing of a consent. Minimum of 7.5 ml peripheral blood was obtained (1.6 mg EDTA/1 ml of blood as anticoagulant) for the CTC examination once or repeatedly during 6-24 months.

Enrichment, cultivation and detection of CTC

Blood was subjected to the two-step analysis, consisting of the size- based capturing of cells (MetaCell®), the evaluation of cytomorphological parameters of captured and cultured viable cells by the fluorescent microscopy

Table 2. Genes associated with chemoresistance

Resistance to:	Genes associated with chemoresistance:					
Anthracyclines	MRP1	MRP2				
Taxanes		MRP2		MRP7		
Irinotekan/topotekan	MRP1	MRP2	MRP4			
Alkylating agents	MRP1	MRP2				
5-fluorouracil				MRP5		
Platinum derivates		MRP2		MRP5		ERRC1
Metothrexat		MRP2	MRP4	MRP5		
Vinca-alkaloids	MRP1			MRP7		
Multi-drug resistance as defined by MDR1 (P-glykoprotein)						MDR1
Gemcitabine						RRM1/RRMM2

and by the gene-expression analysis (molecular detection).

CTC were enriched by the filtration of the peripheral blood using Metacell® separation tool (MetaCell s.r.o., Czech Republic). Captured cells were cultured *in vitro* under standard conditions (37°C, 5% CO_2) for 3-5 days. Subsequent cytomorphological analysis is based on the characteristics of captured and cultured cells stained by vital fluorescent dyes (Nucblue® Live Ready Probes® Reagent and CellTracker™ Green CMFDA, Thermo Fisher Scientific, USA). The cells were evaluated by standard cytopathologic criteria under the fluorescent microscope (e.g. cells size >15 μm, nucleus size >10 μm, irregular nuclear contour, high nuclear/cytoplasmic ratio, prominent and/or irregular nucleoli, cell proliferation presence, tri-dimensional cell sheets growth). The digital documentation of the captured cells is available for each patient. If the cells with CTC-character were detected, further molecular analysis was provided. CTC were lysed and stored in RLT-buffer with β-mercaptoethanol solution (-20°C).

RNA isolation and cDNA preparation

RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation from the frozen cell lysates (white blood cells (WBC) and CTC) stored in RLT-buffer. RNA quantity and quality has been checked by NanoDrop (Thermo Fisher, U.S.). High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA) for cDNA synthesis using minimum of 100 ng RNA load for single cDNA reaction.

qPCR analysis

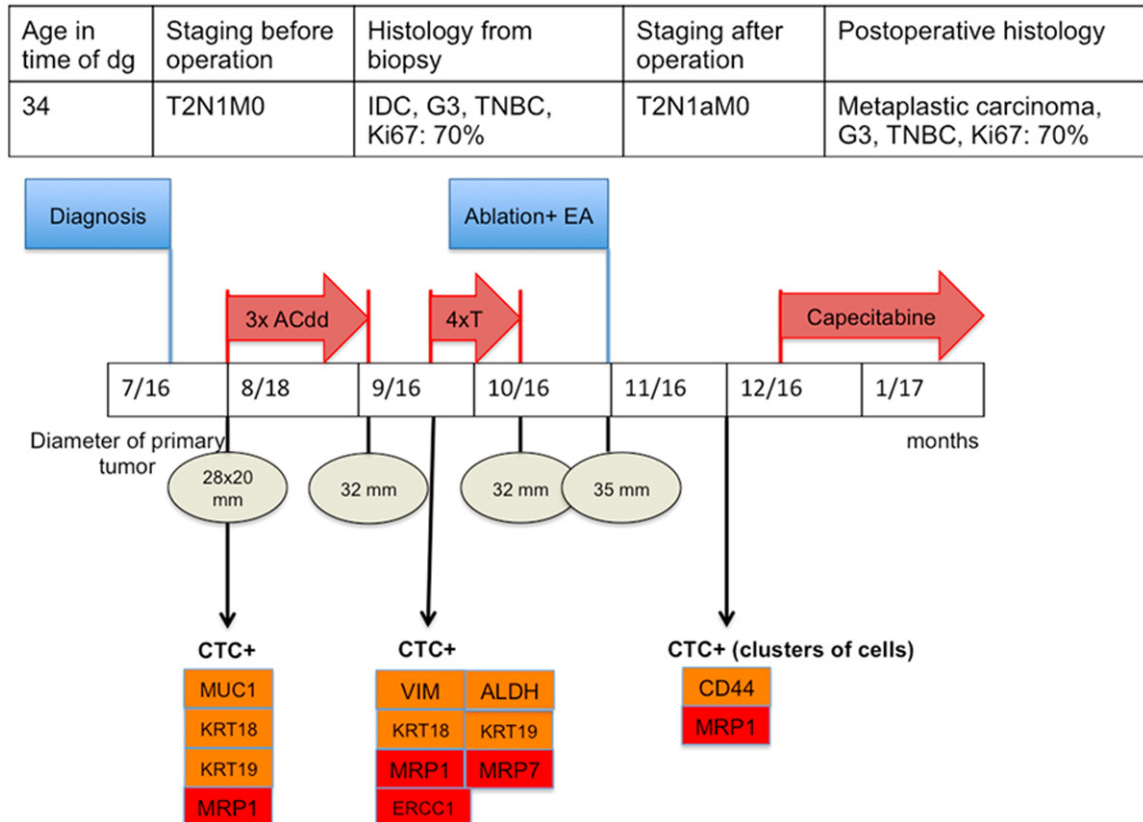The gene expression of tumor-associated (TA) genes (disease specific), stem cell markers and control genes (ACTB) was evaluated. Additionally, the markers of white blood cells (CD45, CD68) were included. Subsequently, expression of genes associated with chemoresistance (CA-genes) was tested. The predictive associations of tested CA-genes MRP1, MRP2, MRP4, MRP5, MRP7, MDR1, ERCC1, RRM1, and RRM2 with the tumor chemoresistance are listed in **Table 2**. TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used for qPCR analysis in the samples analysed by PCR technology system Cobas® 480 (Roche s.r.o., Czech Republic).

The gene expression in particular cell-fractions (WBC, CTC) was evaluated for every patient individually. The gene expression in WBC fraction, CTC-enriched fraction and CTC-enriched and cultured fraction were compared to confirm the cancer cell presence. The qPCR data evaluation was based on $2^{-\Delta\Delta CT}$ methods used to calculate relative changes in the gene expression analysis [35]. Samples with relatively elevated expression of TA-markers (2 and more) in cultured CTC-fraction compared to WBC fraction were evaluated as CTC positive based on gene expression analysis. qPCR results were analysed by the means of GenEx Professional software (MultiD, Sweden) enabling multifactorial comparison (e.g WBC vs. CTC) applying Mann- Whitney test (P<0.002) in particular patients.

Clinical interpretation

The CTC-test result forms report on information about the presence/absence of CTC including the following statements: CTC presence was confirmed by the cytomorphological evaluation (YES/NO), CTC presence was confirmed by the elevated gene expression of the following

Age in time of dg	Staging before operation	Histology from biopsy	Staging after operation	Postoperative histology
34	T2N1M0	IDC, G3, TNBC, Ki67: 70%	T2N1aM0	Metaplastic carcinoma, G3, TNBC, Ki67: 70%

Figure 1. CTC monitoring during neoadjuvant chemotherapy in a patient with breast cancer. AC: doxorubicin + cyclophosphamide, dd: dose dense, T: paclitaxel, CTC+: CTC positivity, EA: axilla exenteration, dg: diagnosis, G: grade, TNBC: triple negative breast cancer, IDC: invasive ductal carcinoma, markers of stem cells: CD44/CD24, VIM (vimentin), ALDH (aldehyddehydrogenase), markers of epithelial cells: KRT18/19 (keratins), MUC1 (mucin), markers of chemoresistance: see **Table 2**.

genes: e.g. KRT18, KRT19, MUC1, CD24, HER2, ESR. The chemoresistance of CTC may be predicted by the elevated gene expression of the following genes: e.g. MRP2, MRP7. The combination of MRP2 and MRP7 may indicate a resistance to taxanes. MRP2 itself indicates a resistance to platinum-derivatives. MRP7 could be involved into a resistance against vinca-alkaloids too. Repeated measurements enable monitoring of dynamic changes on CTC in time.

Results

Clinical implementation of CTC-examination: Prediction of disease response to neoadjuvant chemotherapy (NACT) in a patient with BC (Case report 1)

Hypothesis: CTC monitoring during NACT may help to predict the early failure of the cancer therapy.
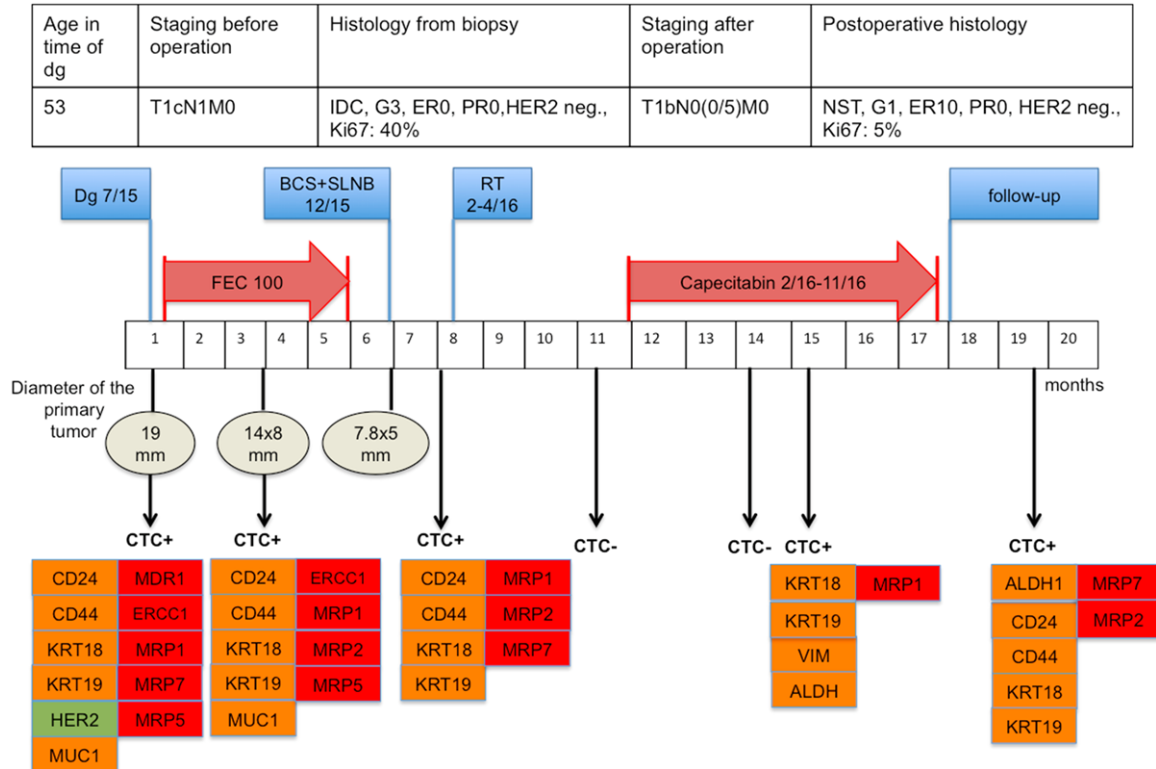
State of the art: To reveal non-responders based on clinicopathological parameters are

not entirely possible. Conversely, CTC properties and, in particular the sensitivity to various cytostatics could be a predictor of the treatment response. The early shift of the chemotherapy regimen based on the evolving chemoresistance could boost the treatment efficacy. The characteristics of the primary disease usually do not correlate with the presence of CTC [36]. The detection of CTC provides additive prognostic and predictive information. The disease progression and the presence of CTC with the mesenchymal characteristics [37] could be the reason for the premature termination of NACT and the indication of a surgery. Whether the presence of CTC with mesenchymal features (stem cells like) can negatively influence the prognosis of the patients is not clear.

Predictive effect of pathologic complete remission is not exclusive, too [38].

Patient report (1): the response to NACT in triple negative BC (TNBC) patient (34 years old,

Age in time of dg	Staging before operation	Histology from biopsy	Staging after operation	Postoperative histology
53	T1cN1M0	IDC, G3, ER0, PR0,HER2 neg., Ki67: 40%	T1bN0(0/5)M0	NST, G1, ER10, PR0, HER2 neg., Ki67: 5%

Dg 7/15 — BCS+SLNB 12/15 — RT 2-4/16 — follow-up

FEC 100 — Capecitabin 2/16-11/16

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 months

Diameter of the primary tumor: 19 mm — 14x8 mm — 7.8x5 mm

CTC+ — CTC+ — CTC+ — CTC- — CTC- — CTC+ — CTC+

CTC+:
CD24	MDR1
CD44	ERCC1
KRT18	MRP1
KRT19	MRP7
HER2	MRP5
MUC1	

CTC+:
CD24	ERCC1
CD44	MRP1
KRT18	MRP2
KRT19	MRP5
MUC1	

CTC+:
CD24	MRP1
CD44	MRP2
KRT18	MRP7
KRT19	

CTC+:
KRT18	MRP1
KRT19	
VIM	
ALDH	

CTC+:
ALDH1	MRP7
CD24	MRP2
CD44	
KRT18	
KRT19	

Figure 2. Indication of "additional" adjuvant therapy in a breast cancer patient with persisting CTC. CTC positivity: CTC+, CTC negativity: CTC-, FEC: fluorouracil, epirubicin, cyclophosphamid, RT: radiotherapy, BCS: breast conserving surgery, SLNB: sentinel lymphatic node biopsy, dg: diagnosis, IDC: invasive ductal carcinoma, G: grade, markers of stem cells: CD44/CD24, ALDH (aldehyddehydrogenase), VIM (vimentin), markers of epithelial cells: KRT18/19 (keratins), HER2: human epidermal growth factor receptor, MUC1 (mucin), markers of chemoresistance: see **Table 2**.

stage II) has been monitored. Tumor size was 28 mm at the beginning of the NACT; ultrasound examination described several pathological lymph nodes. CTC were present before NACT had started (**Figure 1**).

CTC displayed the expression of these TA-genes: MUC1, KRT18, KRT19 and CA-gene MRP1. After the 3rd therapy cycle with anthracycline (AC regimen), no therapeutic effect was observed by the ultrasound examination. CTC test was positive again and the level of tumor cells resistance spread (expression of MRP1, MRP7 and ERCC1 was elevated). Expression of MRP7 is associated with the prediction of taxane chemoresistance. Nevertheless, the patient received 4 cycles of paclitaxel in a weekly mode. According to the ultrasound imaging, the tumor size remained at 32 mm.

Subsequently, NACT was terminated and the patient was indicated for a surgery. The final histology described a metaplastic carcinoma (35 mm in diameter). The postoperative blood test detected clusters of CTC. The elevated expressions of keratins were no longer demonstrated but CD44 positive cells were present.

To be discussed: Due to the existing anthracyclines resistance (MRP1), the age of patient and the adverse outcome of NACT, the patient continues with the adjuvant capecitabine therapy (therapy choice is discussed below in the next case report).

Clinical implementation of CTC-examination: indication of "additional" adjuvant therapy (AT) in residual disease in a patient with BC (Case report 2)

Hypothesis: CTC molecular analysis during AT may help to predict the therapy efficiency and failure.

State of the art: AT administered after the removal of the primary tumor is one of the most difficult treatment strategies. The indication for the adjuvant chemotherapy (ACT) lacks person-

Age in time of dg	Staging of the disease	Histology
29	pT1c multi(3)pNO(0/2) M0	TNBC, G3, Ki67: 60%
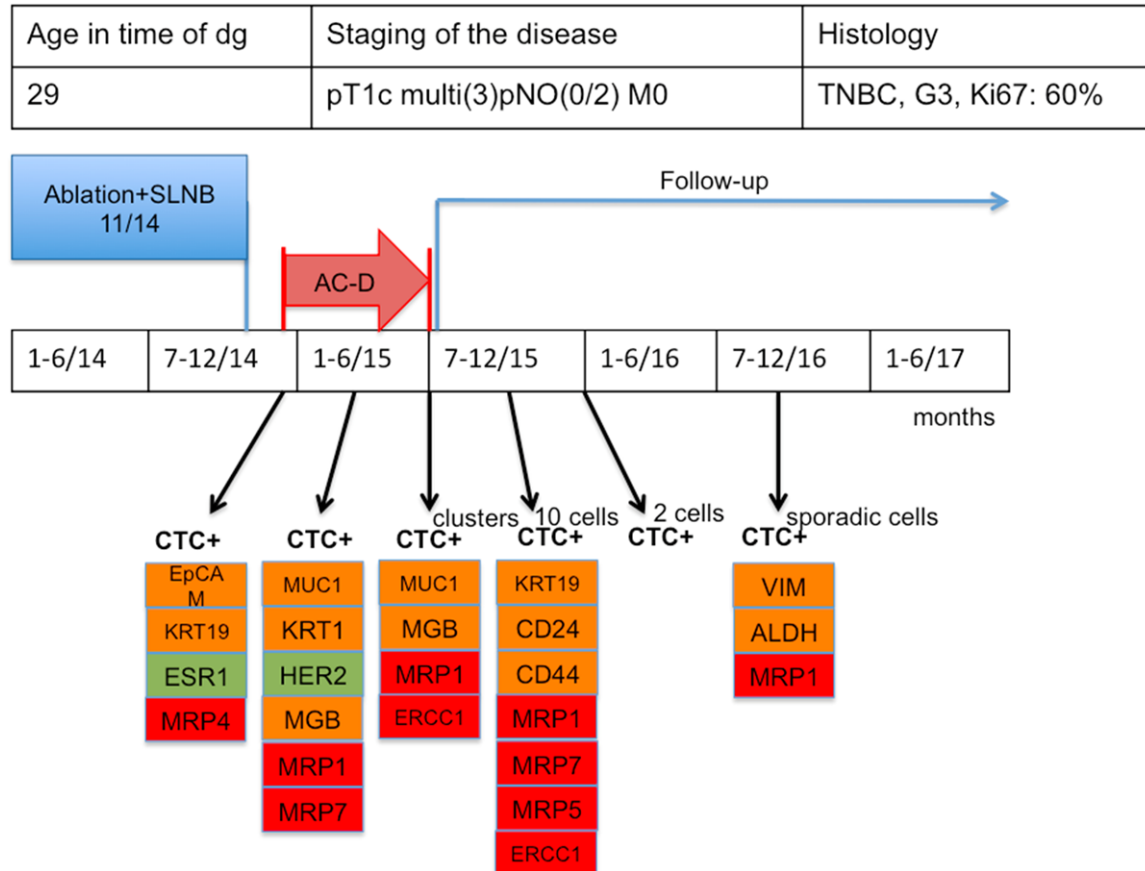
Figure 3. CTC monitoring during adjuvant therapy and in follow up period in a breast cancer patient. CTC positivity: CTC+, AC: doxorubicin + cyclophosphamide, D: docetaxel, SLNB: sentinel lymphatic node biopsy, dg: diagnosis, TNBC: triple negative breast cancer, G: grade, markers of stem cells: CD44/CD24, ALDH (aldehyddehydrogenase), VIM (vimentin), markers of epithelial cells: KRT18/19 (keratins), HER2: human epidermal growth factor receptor, ESR1: oestrogen receptor gene, MUC1 (mucin), MGB: mammaglobin B, markers of chemoresistance: see **Table 2**.

alization and endangers the patient with an unnecessary treatment and a possible ineffective therapy. The correct indication of the correct therapy requires further identification of the residual disease.

There is a lack of data for ACT indication beyond the standard length of the therapy. Create-X study [39] is the only study addressing this question, however, with many questions regarding not only the primacy of data [40, 41]. Nevertheless, an "additional" treatment strategy is requested in the clinical practice.

Molecular typing of TNBC divided this diagnosis into several subtypes with a different prognosis [42]. Treatment guidelines of TNBC have not accepted this fact yet, although the chemotherapy response differs in individual subtypes of TNBC [43]. The need for predictive markers in

TNBC is therefore more than obvious and possible use of CTC is definite.

Patient's report (2): A case report of TNBC patient (44 years old, stage I) undergoing the additional ACT after the completion of NACT is reported (**Figure 2**). The ACT indication was based on the CTC persistence and primary disease residuum.

The first blood sample was tested before the start of NACT. Keratins (KRT18, KRT19), mucin (MUC1), human epidermal growth factor receptor (HER2) and MRP1 genes were overexpressed in the CTC-enriched fraction (for more details about CTC during NACT see **Figure 2**).

CTC persisted postoperatively, as well as their chemoresistant character. Although no CTC were present after RT, additional ACT with capecitabine was started. The presence of CTC

after the 4th capecitabine cycle was not confirmed. After the 6th capecitabine cycle CTC were detected again, furthermore the cells exhibited clustering and overexpressed markers associated with the mesenchymal character: vimentin (VIM) and aldehyddehydrogenase (ALDH1). We assume that the super-selection of the aggressive clone arose during the course of the capecitabine therapy. The expression of HER2 was seen only at the beginning of NACT. Because of the persistent sensitivity to the current treatment we continued up to 8 cycles of the capecitabine therapy. The patient is currently being monitored without any therapy and without any disease relapse.

To be discussed: In the presented case report we can demonstrate the aggressiveness of the tumor defined by persistent CTC long after the completion of the primary therapy and the possible therapeutic strategy of "watchful waiting" with the administration of systemic therapy apart from the completion of primary treatment. The indication of capecitabine according to the Create-X study is not entirely definite, as well as its inclusion into the AT treatment scheme in the period after the RT and in small T1b tumors. On the other hand, we know that the release of substantial quantities of CTC occurs early in tumors under 3 mm in the diameter [44]. Based on the observations in mice, the clusters of CTC have under observations in mice, 23-50 × higher metastatic potential, their presence thus predicts the ability of cells to establish secondary lesions [45].

Clinical implementation of CTC-examination: observation after AT in a patient with TNBC (Case report 3)

Hypothesis: CTC molecular analysis after AT may help to predict disease relapse.

CTC presence after the tumor resection and/or after the completion of AT predicts higher risk of the disease relapse. Since none of the CTC-predictive use has been reliably demonstrated yet, there is the question of how to deal with the prognostic information offered by a regular CTC examination [46].

Patient's case (3): We enclose results of the postoperative CTC monitoring of a patient with TNBC (29 years old, stage I). CTC tests were provided during AT and subsequently in a follow-up period (**Figure 3**).

As shown in the figure, the presence of CTC with epithelial origin was detected during the AT course. Although the primary tumor was TNBC, CTC overexpressed oestrogen receptor (ESR) and HER2. Before the last docetaxel cycle (07/2015) during AT, we observed clustering of CTC and ER/HER2 lost. In the samples taken in 09/2015, 12/2015 and 09/2016 the number of CTC decreased and the characters of the cells changed from epithelial to mesenchymal (increased expression of VIM and ALDH). After the therapy completion, CTC remained resistant to antracylines (epression of MRP1) for the rest of the time. We also registered elevated ERCC1 expression, which seems to be connected with stem cells like phenotype of CTC quite often as published in 2016 by Kasimir-Bauer et al. [47].

To be discussed: The persistence of the low amount of CTC with the signs of the stem cells and MRP1 resistant behaviour during the follow-up period is reported, but the patient is still in remission clinically.

Clinical implementation of CTC-examination: CTC monitoring after AT and during metastatic disease in a patient with HER2+ BC (Case report 4)

Hypothesis: CTC molecular characterisation during the metastatic disease follow up period may help to predict the therapy indication.

State of the art: CTC-positivity and HER2+ are both negative prognostic markers in BC. Together with HER2 discordance between the primary tumor and CTC (in studies 15%-35%), the disease becomes more aggressive and worse from the prognostic point of view [29, 48, 49]. The change of CTC phenotype is spontaneous [50] and behaviour of HER2+ CTC (proliferative potential) is different from those of HER2 negative (resistance to targeted therapy).

We observed the presence of CNS metastases in a patient (39 years old, stage II) with HER2+ locally advanced BC. CNS metastases were detected 11 months after the completion of trastuzumab therapy (**Figure 4**).

The patient started the NACT in 2014, the tumor responded to anthracyclines based therapy well but the effect of taxanes and trastuzumab was quite poor. CTC expressed HER2 at the beginning of the disease therapy, but not later during the taxane-based therapy.
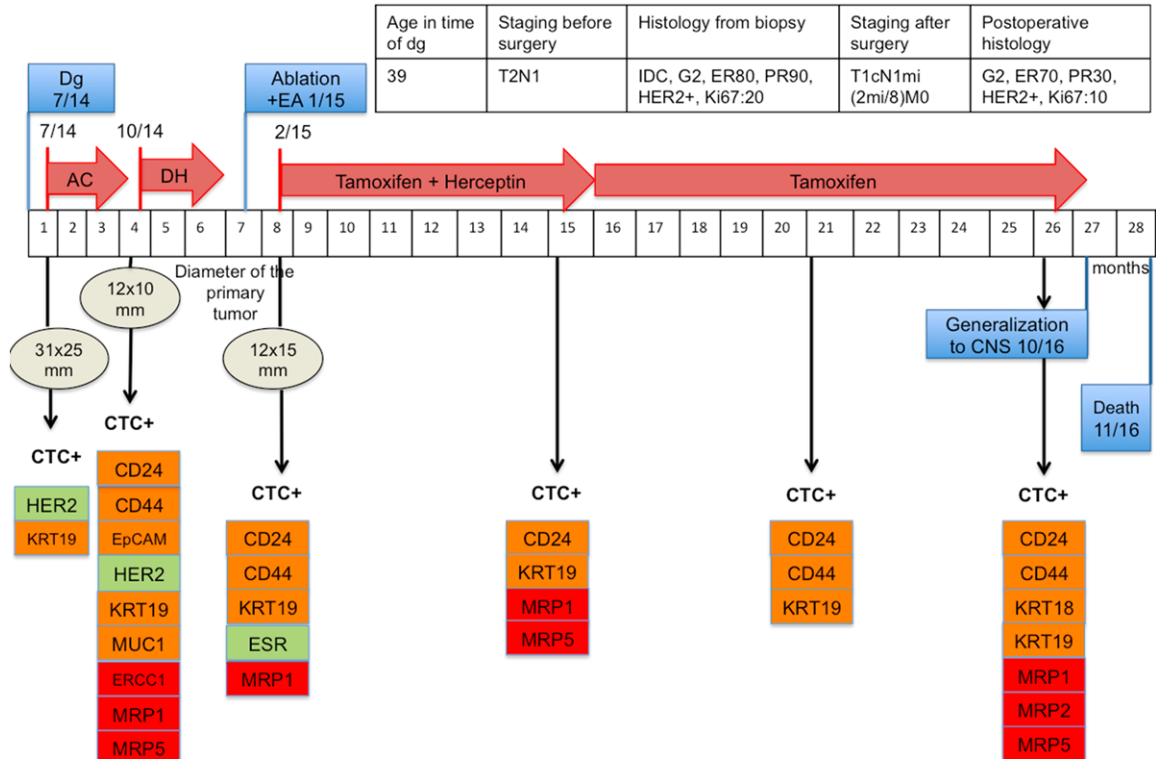
Figure 4. CTC monitoring in a patient with HER2- positive breast cancer. CTC positivity: CTC+, AC: doxorubicin + cyclophosphamide, DH: docetaxel + herceptin, EA: axilla exenteration, dg: diagnosis, ER: oestrogen receptor, PR: progesteron receptor, HER2: human epidermal growth factor receptor, G: grade, CNS: central nervous system, markers of stem cells: CD44/CD24, markers of epithelial cells: KRT18/19 (keratins), EpCAM (Epithelial Cell Adhesion Molecule), MUC1 (mucin), markers of chemoresistance: see **Table 2**.

During the AT (tamoxifen+ herceptin) CTC positivity was confirmed regularly. HER2+ CTC were found during AT with trastuzumab. Expression of ESR was detected in only two of CTC postoperative samples (02/2015 and 04/2015).

The elevation of CTC count and chemoresistance had been documented again before the disease progression and brain metastasis were detected. The expression of KRT18 and CD44 was elevated. Shortly after the trastuzumab therapy completion, CTC expressing HER2 were not present anymore. The patient's death occurred very quickly after the diagnosis of brain metastases.

To be discussed: One could discuss the possibility of the re-administration of anti-HER2 therapy in the case of HER2+ CTC at the time of the brain metastases development. The effect of tamoxifen treatment could be redundant also as CTC did not express ESR. Such decisions do not reflect the existing recommendations and could be only used in clinical trials.

Clinical implementation of CTC-examination: CTC-testing after resection of metastases and early prediction of disease relapse in a patient with metastatic CRC

Hypothesis: CTC-examination including the chemoresistance profile analysis could help in the therapy indication in the metastatic disease course.

In patients with CRC and isolated metastatic liver disease, the presence of CTC has already been examined in several studies. The observation period included the time before and/or after the resection or radiofrequency ablation (RFA) [51-53].

According to the type of the detection method, CTC were found in 10-30% of patients before surgery, 29-50% of patients during the surgery and in 5-28% of patients after the surgery. The presence of CTC in the time during or after the surgery had a prognostic significance.
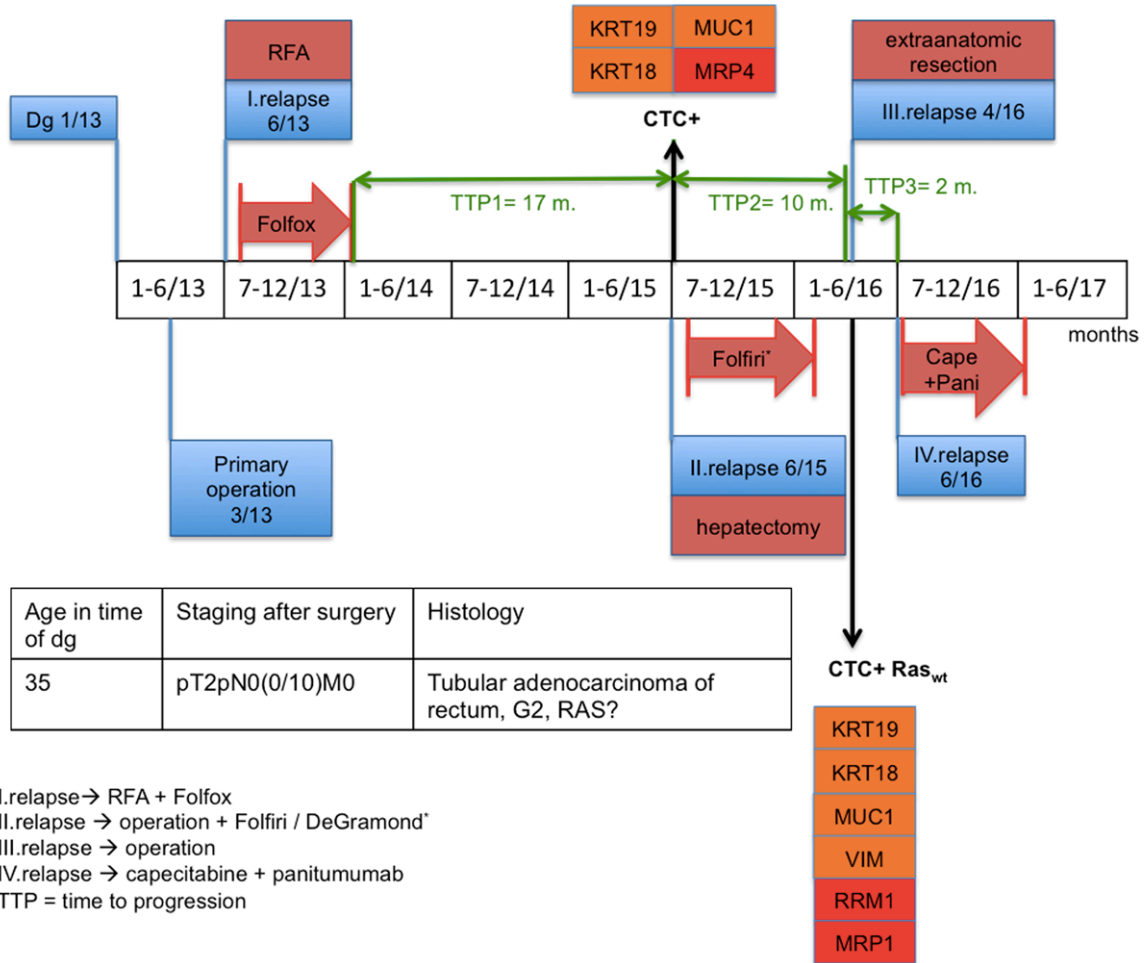
Figure 5. CTC-testing after resection of metastases in a patient with metastatic colorectal cancer. CTC positivity: CTC+, dg: diagnosis, G: grade, RAS: group of oncogenes, wt: wild type, RFA: radiofrequency ablation, m: months, cape: capecitabine, pani: panitumumab, FOLFOX/FOLFIRI: chemotherapy regimens (see main text), KRT18/19 (keratins), MUC1 (mucin), VIM (vimentin), genes of chemoresistance: see **Table 2**.

The monitoring of CTC in real-time and the observation of their dynamic behaviour would help to detect early the disease relapse. Properties of CTC could also help to predict an individual risk of disease relapse [54] and to choose the therapy after the resection of liver metastases. The benefit of the targeted therapy in the adjuvant indication has not been demonstrated in CRC yet [55-58]. The presence of CTC with RAS wild type (RAS_{wt}) properties could change this situation by positive selection of CRC patients.
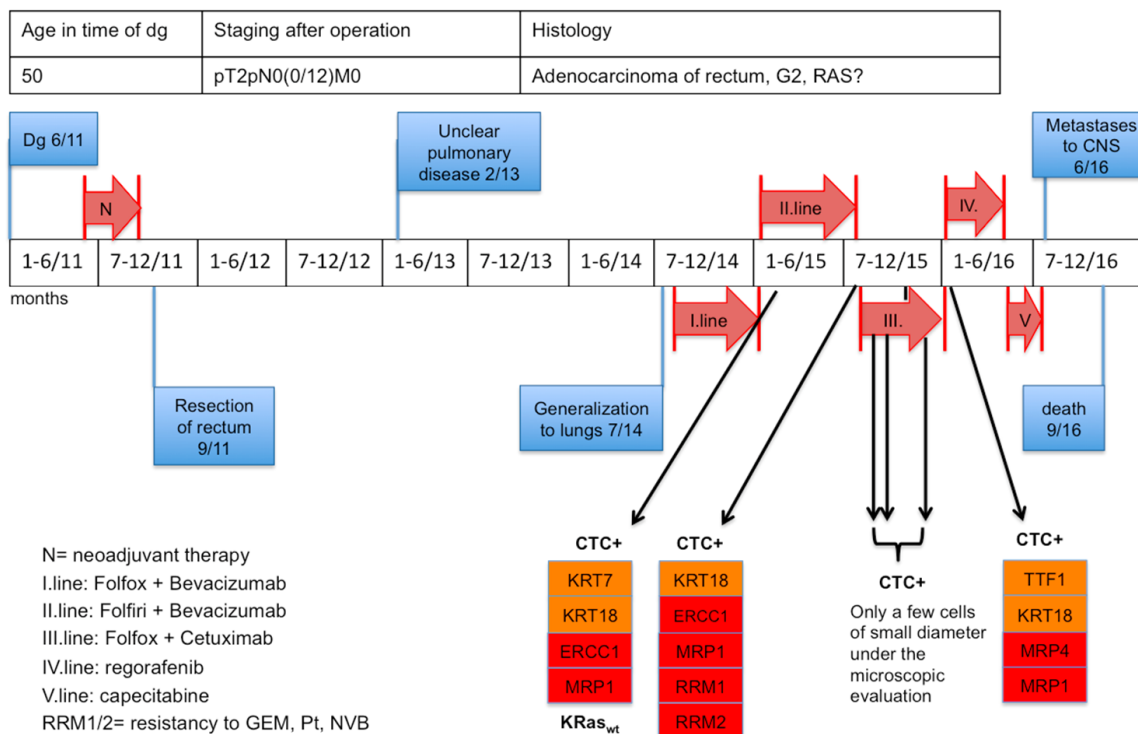
Patient's case (5): The patient (35 years of age) with CRC was undergoing a surgery because of adenocarcinoma of rectum. The postoperative staging was T2N0(0/10)M0, the status of RAS could not be examined because of heavy DNA fragmentation. Short time after the surgery,

liver metastasis developed in the left liver lobe. The tumor was cured by RFA and ACT (FOLFOX regimen) (**Figure 5**).

18 months later the second liver relapse appeared. Liver metastasis was resected again. The patient was secured with a systemic therapy (FOLFIRI) and blood collection for CTC examination was indicated. The results were showed CTC presence by molecular analysis, higher expression of keratins and MUC1 was confirmed, no mesenchymal markers were detected. A relatively small number (units of cells) of CTC could be the reason for quite a long time to the next progression (TTP2).

The third liver relapse developed after 10 months in 04/2016 and CTC test was positive again. Not only liver metastases but also CTC

Age in time of dg	Staging after operation	Histology
50	pT2pN0(0/12)M0	Adenocarcinoma of rectum, G2, RAS?

Figure 6. Assessment of KRAS mutation status from CTC in a patient with metastatic colorectal cancer. CTC positivity: CTC+, dg: diagnosis, G: grade, RAS: group of oncogenes, wt: wild type, CNS: central nervous system, FOLFOX/FOLFIRI: chemotherapy regimens (see main text), KRT18/19 (keratins), TTF1 (Thyroid transcription factor 1), genes of chemoresistance: see **Table 2**.

were tested for the presence of RAS mutations with a negative result (RAS_{wt} was confirmed). The expression of VIM, clustering of CTC and a high number of CTC were signs for the high disease relapse risk.

The 4th relapse developed in 2 months (TTP3). Because the patient refused any additional chemotherapy, an attempt was made by another liver resection, but with a short effect only. This patient was treated with anti-EGFR monoclonal antibody and capecitabine from 07/2016 to 02/2017.

To be discussed: The disease volume and KRAS status could be controlled by monitoring of CTC after the surgical removal of metastases.

Clinical implementation of CTC-examination: assessment of KRAS mutation status from CTC in patient with metastatic rectal adenocarcinoma

Hypothesis: CTC could present a relevant real-time information source displaying mutational status for genes relevant in the therapy indication process.

State of the art: As we know from the clinical trials, the wild form of KRAS oncogene ($KRAS_{wt}$) is associated with the sensitivity to anti-EGFR therapy, especially in the tumors of the left colon. According to some published studies, the discordance in the state of KRAS in comparison with the primary tumor and metastases is relatively small [59-61]. Likewise, relatively good correspondence in the state of KRAS between the primary tumor and peripheral blood is the reason for the effect of anti-EGFR therapy regardless the fact whether the result of KRAS is based on analysis of the primary tumor or CTC [62, 63].

The problem may occur in advanced lines of treatment because of previous therapy, which can cause super-selection of aggressive tumor clones. Discrepancies at various disease levels (primary tumor, metastasis, blood) could be striking [64]. Another possible benefit of RAS oncogenes or other genes determination from CTC is in cases, in which we lost the option to analyse RAS directly from the primary or secondary tumor.

Patient case (6): A case of a patient (50 years old, stage III) with rectal adenocarcinoma with unknown status of KRAS gene, because of low amount of primary tumor material, is presented (**Figure 6**).

The bulk in the left lung was discovered one year after the NACT therapy (02/2013). The patient has been under observation only because of bulk low diameter and the absence of other signs of an active disease. In 07/2014 lung metastases were confirmed by PET/CT and CTC were detected in the blood. The patient was treated with FOLFOX and bevacizumab in the 1st line and FOLFIRI and bevacizumab in the 2nd line but with only 6 and 3-month lasting effect. CTC examined after FOLFOX were resistant to oxaliplatin.

Before the initiation of the 3rd line of the therapy a biopsy from a newly discovered tumor mass in the liver was executed, but tumor cells were not aspirated. The analysis of KRAS was provided, based on CTC-material with the result of $KRAS_{wt}$. Nevertheless, combined FOLFOX and cetuximab therapy failed again. Only relatively small cells with several cancerous morphologic features were detected in the blood after the therapy completion. CTC expression profile was not done because of small amount of RNA.

We explain the therapeutic failure of the anti-EGFR therapy by tumor heterogeneity and by the administration of two previous therapy lines, which might cause the selection of chemoresistant cells subset (MRP1 and MRP4 expression).

The disease progressed macroscopically and new lesions in bones were discovered in 11/2015. We treated the patient with regorafenib and capecitabin in the next two lines but without any significant effect. The patient died in 9/2016 because of new CNS lesions. CNS metastases are not typical among CRC patients, and their presence explains the aggressiveness of the disease.

To be discussed: To influence the prognosis of the patient at the stage of generalization, the early treatment initiation is critical, but the verification of pulmonary focus (07/2014) could not be done, unfortunately. The liquid biopsy in such a case could replace screening, focused on the disease relapse verification. The molecular analysis of CTC including KRAS status analysis should be more perspective at the beginning of the disease. The effectiveness of anti-EGFR therapy was certainly affected by the previous treatment and by the chemoresistance of the disease, which was documented by examination of CTC. Increased EGFR expression could also be the cause of non-effect of regorafenib as a possible escape mechanism of the tumor cells in CRC patient [65]. The data correlating the status of RAS mutations are usually obtained from patients receiving the first line therapy [66, 67], however it was shown that the monitoring of CTC could be relevant in the advanced lines of therapy, too [68].

Clinical implementation of CTC-examination: strategy of using CTC for the palliative treatment guidance in a patient with NSCLC

Hypothesis: CTC-examinations could be used for EGFR mutation detection during the therapy course in a patient with NSCLC.

State of the art: The dynamic changes of CTC could reflect the prognosis of patients with NSCLC [69]. The chemotherapy efficiency decreases with the sequential selection of the chemoresistant tumor clones and its success can be a guarantee only by using drugs with the new mechanism of the effect, which could target on the slowly dividing cells and/or restoring the sensitivity of the tumor cells to cytostatics. The examination of CTC chemoresistance is one way of how to better choose the potentially effective cytostatic in palliative care. The mutational analysis of CTC may offer new information on EGFR-mutational status, identifying T790M mutation associated with anti-EGFR treatment resistance.

Patient's case (7): The case of 47-year old patient with stage IV NSCLC treated with combined carboplatin and pemetrexed therapy in the 1st line is presented. Her disease had the character of adenocarcinoma without mutations in genes EGFR, KRAS, NRAS, BRAF and ALK fusion was also not found in the primary tumor. The therapy was conducted from 01 to 05/2016. The examination in 02/2016 showed the presence of CTC with the expression of TA-associated genes EpCAM, MUC1, KRT18 and KRT19. CTC showed resistance to platinum

(ERCC1) and cross-resistance to several other antineoplastic agents (MRP1).

CT (computer tomography) scan from 05/2016 showed a mild non-effect of the therapy. The control blood test was carried out at the same time, the characterization of CTC changed partly, and the expression of VIM was newly verified. The disease developed more multi-resistant cells (expression of MRP1, MRP2, MRP4, MRP7, and ERCC1). Based on this result and based on the preserved sensitivity of the disease on derivatives of 5-fluorouracil (MRP5 expression has not been proven) and gemcitabine (expression level of RRM1 or RRM2 was not elevated), we indicated the treatment with gemcitabine and capecitabine in the 2nd line.

CT scan from 09/2016 showed a slight progression of a one pulmonary node but also the regression of tumors in other locations. An unresponsive focus was subsequently irradiated and after the completion of RT (09-10/2016) we continued with palliative treatment in the mentioned scheme till 12/2016. The control CT scan unfortunately revealed further bilateral progression of lung focuses. Despite of this result we declare the effect of the second-line treatment lasting for 6 months as successful.

To be discussed: CTC-assisted therapy supplemented by chemoresistance testing may contribute to a better therapeutic effect.

Clinical implementation of CTC-examination: typing of tumors with unknown primary site (C80) or duplicate tumors

Hypothesis: CTC-examination could be used for diagnostics of tumors with unknown origin or for the differential diagnosis in patients with duplicate tumors.

State of the art: CTC could be beneficial in tumors of the unknown primary site (diagnosed as C80). The inter-individual heterogeneity or the tumor dedifferentiation delimitate successful typing of known origin tumors as well as of C80 [70]. Detailed analysis of DNA allows to find deviations and mutations of genes involved to the pathogenesis of C80 [71], which could be the aims for the targeted therapy [72]. CTC represent a possible extension of the knowledge obtained from the tissue biopsies [73].

Patient's case (8): A 57-years old patient with duplicate CRC and PC, CTC examination was indicated to obtain prognostic information and to identify the type of CTC. CRC was resected; the post-operative stage was pT3pN0(14)M0, microsatellite stable (MSS). The PC stage T3b-N0-1, GS 3+4 was planned to examine by using choline- PET/CT. We discussed the need of AT in CRC. We considered both cancers as potentially aggressive; CRC because of their biological behaviour, PC because of the extent of the disease.

Detected CTC overexpressed following TA-genes: KRT18, KRT19, VIM, ALDH, VEGF, AMACR. The subset of genes confirmed presence of the cells with epithelial origin (keratins), but the elevation of stem cell markers (VIM, ALDH1) was also demonstrated. Additionally VEGF expression supporting the tumor angiogenesis was elevated. The cells were exhibiting morphological features of the cells found in the patients with CRC, but the elevated expression of the AMACR gene could be ascribed to the cells of prostate origin. We concluded that probably the both cell types from both tumor types were present in the patient's blood. The genes associated with the chemoresistance to anthracyclines (MRP1) and platinum (ERCC1) were detected.

We also indicated Oncotype DX Colon Cancer (Genomic Health, USA) examination with the result of the middle to high risk of the disease relapse according to the molecular print of the primary colon cancer (score of recurrence 39).

As RT of PC was planned, we recommended capecitabine as adjuvant monotherapy for CRC and dipherelin as the primary neoadjuvant treatment for prostate cancer.

To be discussed: The CTC examination helped us to distinguish the risk of relapse in two different malignant diseases. Stage II CRC does not always require ACT. The liquid biopsy could predict the need of the post-operative therapy in such cases. In comparison to molecular assays targeting the primary disease, the liquid biopsy offers a real-time monitoring of the CTC volume in time.

Discussion

The clinical evidence for the predictive value of CTC is still limited. Our two-step detection pro-

tocol combining a size-based filtration with the both cytomorphological and molecular characteristics of CTC may identify CTC in patient samples, where they cannot be detected by other methods (e.g. EpCAM-based separations). In the reported CTC positive samples (24/34 i.e. 70.6%) EpCAM expression has been confirmed in 2 samples (8%) only, expression of KRT 18/19 in 23 samples (95.8%) and MUC1 in 7 samples (29.2%). The changes in the number of CTC in responders compared to non-responders suggest that CTC properties are different in the patients with the same disease undergoing the same treatment.

The highest possibility for the CTC clinical implementation is in the palliative indication. The liquid biopsy- navigated therapy based on the detection of certain types of mutations in NSCLC patients is already part of a clinical care. We expect a similar use of CTC-testing in other diagnosis (e.g. the determination of RAS status in CRC or ARV7 in CRPC patients) and in the cases of the primary disease, where the tumor tissue is not approachable for a biopsy verification.

Considering the chances for CTC implementation in AT, monitoring of CTC in the patients in remission after the completion of the primary treatment is the only way how to actively intervene to the course of this period. It is evident that a disease relapse may occur even years after the primary diagnosis. The persistence of CTC in the blood of the patients in remission after primary treatment significantly increases the risk of a relapse. Long observational periods increase the already limited budgets for the cancer treatment. The liquid biopsy could be a promising inexpensive screening method (in the context of a comprehensive pharmaco-economic assessment) [74].

To sum up, in all the presented case reports it has been shown that the aggressiveness of the disease may be defined by the persistent CTC long after the completion of the primary therapy (e.g. NACT, AT). Subsequently, possible therapeutic strategy of "watchful waiting" combined with the administration of systemic therapy apart from the completion of the primary treatment could be of a help.

Considering the results of the CTC-chemoresistance test (e.g. resistance to the anthracyclines defined by MRP1), the patients could overcome the "watchful waiting" periods with a support of an "additional" AT, e.g. by capecitabine administration after NACT in BC. Similarly, if low amount of CTC with signs of the stem cells in the follow up period is reported, the metronomic strategy of therapy could be considered.

Another situation has been described when HER2+ CTC were detected in the patients after the terminated anti HER2-therapy. One could discuss the possibility of a re-administration of the anti-HER2 therapy, especially at the time of the brain metastases development.

Our case reports show, monitoring of CTC, not only in BC but also in CRC and NSCLC, could control the disease volume after the surgical removal of metastases. In the last two mentioned cases, CTC could be used for molecular subtyping of the tumor, which is in CRC and NSCLC a necessary condition which allow the anti-EGFR treatment indication.

In all of the cases the CTC-assisted therapy, supplemented by the chemoresistance testing may contribute to a positive therapeutic effect. In comparison to the molecular assays targeting the primary disease, the liquid biopsy offers real-time monitoring of CTC volume in time.

Using CTC in the context of the disease diagnostic in tumors of unknown primary site or in the patients with a duplicate tumor could accelerate the therapeutic management of the cancer patients in general.

Based on the data presented, we assume, that the liquid biopsy could significantly improve the ability to monitor the malignant disease, to predict the treatment efficacy and to provide an additional base for the complementary use of CTC in concordance with conventional histology in the future.

Disclosure of conflict of interest

None.

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