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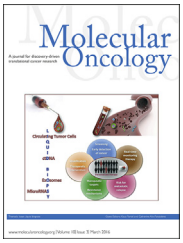

Review

Gene expression profiling and DNA methylation analyses of CTCs☆

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ABSTRACT

A variety of molecular assays have been developed for CTCs detection and molecular characterization. Molecular assays are based on the nucleic acid analysis in CTCs and are based on total RNA isolation and subsequent mRNA quantification of specific genes, or isolation of genomic DNA that can be for DNA methylation studies and mutation analysis. This review is mainly focused on gene expression and methylation studies in CTCs in various types of cancer.

Abbreviations: aCGH, array-comparative genomic hybridization; *BRMS1*, Breast Cancer Metastasis Suppressor-1; CEA, carcinoembryonic antigen; *CK-19*, Cytokeratin 19; *CST6*, cystatin M; CTC, Circulating Tumor Cells; ctDNA, circulating tumor DNA; EpCAM, Epithelial cell adhesion molecule; *HER-2*, human epidermal growth factor receptor 2; hMAM, human mammaglobin; *MAGE A3*, Melanoma-associated antigen 3; MBC, metastatic breast cancer; MSP, Methylation specific PCR; MUC-1, Mucin 1, Cell Surface Associated; *PBGD*, Porphobilinogen deaminase; qPCR, quantitative PCR; RNA-Seq, RNA-sequencing; RT-PCR, Reverse transcription PCR; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; *SOX17*, SRY-box containing gene 17.

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1. Introduction

The presence of Circulating Tumor Cells (CTCs) in peripheral blood has been linked to worse prognosis and early relapse in numerous clinical studies and in various types of solid cancers (Plaks et al., 2013; Pantel et al., 2009). Especially after FDA has cleared the CellSearch® system (Jannsen/Veridex, J&J, USA) for metastatic breast, colorectal and prostate cancer, the critical role that CTCs play in the metastatic spread of carcinomas has been widely recognized (Cristofanilli et al., 2004; Lianidou et al., 2013; Zhang et al., 2012).

Far beyond simple detection and enumeration, the molecular characterization of CTCs, may provide real-time information on a patient's disease status. Especially, the identification of specific therapy-related molecular targets on CTCs such as gene expression, chromosomal translocations, or gene mutations expressed on CTCs could offer important information, early on to choose for the correct treatment and moreover explain resistance to established therapies (Lianidou et al., 2013, 2012; Zhang et al., 2012; Sieuwerts and Jeffrey, 2012). The identification of basic molecular pathways of invasion, migration and immune surveillance on CTCs might even contribute to the development of improved therapies in the near future (Korkaya and Wicha, 2013; Becker et al., 2014). The identification of therapeutically relevant targets expressed on CTCs could stratify cancer patients for individual therapies, and a plethora of translational trials now in progress will hopefully provide soon critical data towards the establishment of CTCs use in personalized treatment (Georgoulias et al., 2012; Bidard et al., 2012). This approach is expected to provide in the near future real-time monitoring of tumor evolution and therapeutic efficacy, and has the potential for improved cancer diagnosis and treatment (Ignatiadis et al., 2015).

CTCs molecular analysis represents nowadays a very promising diagnostic field for cancer patients, and a major player in the liquid biopsy area. A lot of international effort is now focused towards the development and perfection of novel systems for CTCs isolation, detection and molecular characterization (Pantel and Alix-Panabieres, 2012; Alix-Panabières and Pantel, 2013). This task is very challenging and demanding, since the amount of available sample for molecular analysis is very limited, while the number of interesting targets to be evaluated on CTCs is constantly increasing, while at the same time it is now clear that CTCs are highly heterogeneous even within the same patient (Powell et al., 2012).

Molecular assays for studying gene expression on CTCs take full advantage of the extreme sensitivity and specificity of reverse transcription quantitative PCR (RT-qPCR) and multiplex RT-qPCR and can be used downstream to many different CTCs isolation systems (Alix-Panabières and Pantel, 2013; Powell et al., 2012; Lianidou and Markou, 2011; Coumans and Terstappen, 2015; Swennenhuis and Terstappen, 2015; de Wit et al., 2015). It is now evident that the expression of specific molecular markers on CTCs as this can be verified by gene expression assays can be extremely important for therapeutic decisions and patient selection, especially if these are indicative of response to specific treatments (Georgoulias et al., 2012; Parkinson et al., 2012). Moreover, the study of DNA methylation on CTCs, using molecular assays like methylation specific PCR (MSP), can provide important information on epigenetic silencing of genes that play a critical role in the biology of metastasis.

In this review we focus on the main principles and clinical applications of molecular assays based on PCR that are used for the detection and molecular characterization of CTCs, at the gene expression and DNA methylation level. In this review we mainly focus on breast, colorectal, prostate, pancreatic and ovarian cancer and not on other types of solid cancers, since the majority of papers based on molecular assays are focusing till now on these types of cancer.

An overview of the molecular assays that are currently being used for CTCs detection and molecular characterization is presented in Figure 1.

2. Molecular assays for studying gene expression on CTCs

Molecular assays for studying gene expression on CTCs take full advantage of the extreme sensitivity and specificity of RT-qPCR. However it is important to note that gene expression analysis of CTCs require special precautions concerning the preanalytical steps; clinical samples should be treated in a way that intact and viable CTCs will isolated in a reasonable time, before proceeding to the isolation of total RNA so that false negative results, due to RNA loss during sample treatment will be avoided (Lianidou and Markou, 2011; Sieuwerts et al., 2011; Andergassen et al., 2013) (Figure 2).

The main advantages (Table 1) of molecular assays for CTCs detection and molecular characterization are the high sensitivity offered by PCR amplification, and the fact that by careful handling the isolated CTCs sample, they can give information both at the RNA and DNA level. Molecular assays offer also the choice of multiplexing, enabling the amplification of many gene-targets at the same time, thus saving precious sample, cost and time of analysis. Moreover, RT-qPCR is characterized by an excellent specificity, in case that primer and probe sequences are very carefully *a-priori* designed. Molecular assays can be quantitative, high throughput and usually require a very small volume for analysis. Measurements are objective, and quantifiable, and are not subjected to personal estimations as in the case of imaging approaches used for CTCs enumeration and molecular characterization (Andergassen et al., 2013; Ignatiadis et al., 2014). Molecular assays can be subjected to a quantifiable quality control system, and have the potential to be distributed in clinical labs in the form of IVD kits, in case that their clinical significance is proven. They can also be easily automated if based on the existing know-how of fully-automated systems for RNA and DNA isolation, and downstream PCR analysis, already used in the routine molecular in-vitro diagnostics field, provided that the first crucial step of CTCs isolation is also standardized.

The main disadvantages of molecular assays for CTCs detection and molecular characterization (Table 1) concern preanalytical issues concerning CTCs stability during sample shipment and storage. Molecular assays currently require

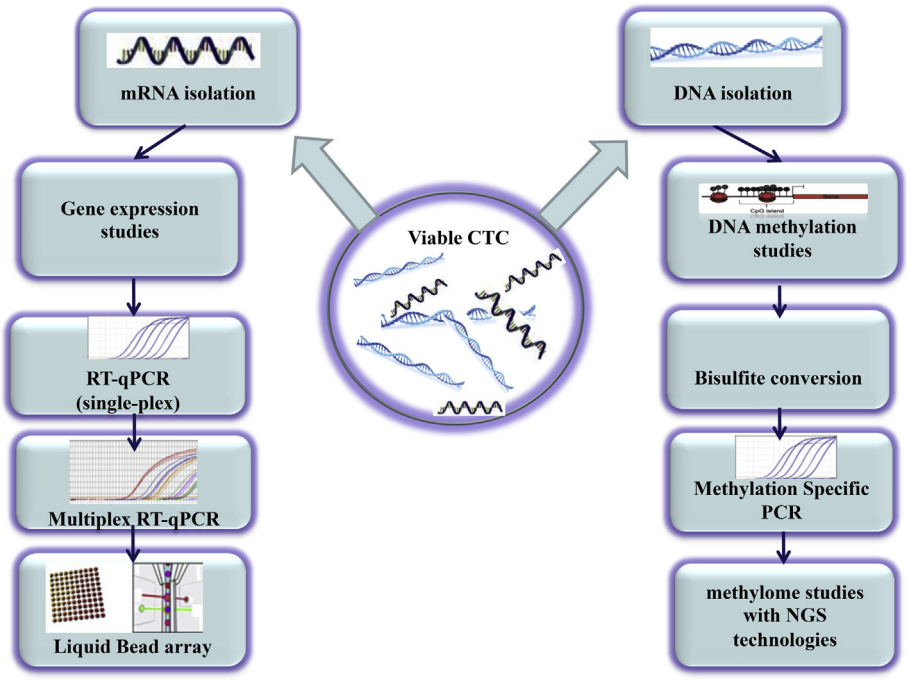

Figure 1 – Overview of molecular methods used for gene expression and methylation analyses of CTCs.

immediate handling of blood samples for CTCs isolation and downstream analysis, a fact that hinders at the moment long-distance shipment of samples to certified centers of analysis. Another major issue is that this type of analysis requires specially designed lab areas to avoid PCR contamination, and different areas for: a) RNA/DNA isolation, b) setting up the PCR reactions, and c) amplification as well as different storage areas for pre-PCR and post-PCR reagents. A major issue is also the fact that through molecular assays, we can only get information on the total number of target transcripts in our sample, not knowing whether these targets are co-expressed in the same cell, or derive from different cell populations, unless single cell analysis is used. Powell et al. have reported a high heterogeneity of CTCs even among the same individuals when they directly measured high dimensional gene expression in individual CTCs without the common practice of pooling such cells (Powell et al., 2012). Since CTCs are highly heterogeneous, even in the same patient, and different CTCs may express the same target at a different level, absolute quantification of CTCs numbers is not feasible with molecular assays, unless analysis is performed at the single cell level.

Recently a novel direct lysis method was developed for studying gene expression on low cell numbers; this highly efficient protocol for RNA extraction, that involves a minimal number of steps to avoid RNA loss, could be essential for low input cell numbers as in the case of CTCs (Le et al., 2015). In this protocol, a lysis solution containing both a non-ionic detergent and bovine serum albumin was used and outperformed a column-based extraction method using a commercial kit.

2.1. *RT-qPCR*

RT-qPCR molecular assays can provide significant and quantitative information on gene expression in an automated, rapid, versatile and cost-effective way (Bernard and Wittwer, 2002). RT-qPCR has been widely used for the detection and molecular characterization of CTCs in a many types of solid cancers up to now. RT-qPCR assays are closed-tube, highly sensitive and specific and can detect and measure minute amounts of specific gene transcripts in minute amounts of clinical samples. In this way RT-qPCR assays are highly appropriate for CTCs analysis. In RT-qPCR assays there is no need for gel electrophoresis, and complicated sample manipulations after the amplification step, while analytical performance and quality control can be validated in detail through a series of standard experiments. Although RT-qPCR is potentially sensitive and specific enough to detect one cancer cell in the presence of more than 10^6 leukocytes, this requires the use of appropriate mRNA markers specific for CTCs (Stathopoulou et al., 2003, 2006). Till now, many research groups have described the usefulness of using RT-qPCR to detect and characterize CTCs (Stathopoulou et al., 2003, 2006; Strati et al., 2011; Xenidis et al., 2009; Yokobori et al., 2013).

2.2. *Multiplex RT-PCR*

Multiplex RT-qPCR assays for CTCs detection and characterization offer a unique advantage since they can amplify and quantify simultaneously many gene targets, using a very small amount of precious CTC-derived nucleic acid sample (Sieuwerts and Jeffrey, 2012; Strati et al., 2011; Fehm et al.,

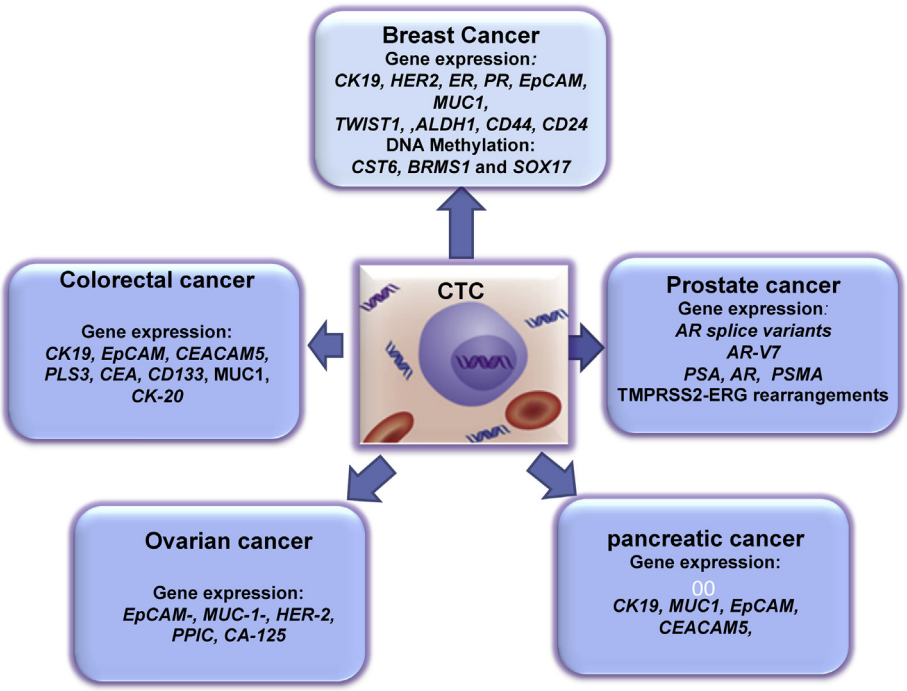

Figure 2 – Gene expression and methylation analyses of CTCs in various types of cancer.

Table 1 – Major advantages and disadvantages of molecular assays for gene expression and methylation analyses of CTCs.

Advantages	Disadvantages
1. Easy to be automated	11. Preanalytical issues
2. Quality control	12. Sample shipment
3. High throughput	13. Immediate analysis of samples
4. Highly sensitive	14. Require specially designed lab areas
5. In silico assay design	15. PCR contamination
6. Low cost	
7. Multiplex assay design	
8. Quantitative	
9. Evaluation of many targets	
10. Splice variants expression	

2009). Few markers provide adequate sensitivity individually, but combinations of markers may produce better sensitivity for CTCs detection. Strati et al. developed a multiplex RT-qPCR assay for cytokeratin 19 (*CK-19*), human epidermal growth factor receptor 2 (*HER-2*), melanoma-associated antigen 3 (*MAGE A3*), and porphobilinogen deaminase (*PBGD*) transcripts in EpCAM positive CTCs of breast cancer patients, that when applied in early breast cancer has shown a superior sensitivity in respect to CellSearch (Strati et al., 2011). The AdnaTest BreastCancer detect kit (Adnagen, Qiagen, Hannover) is a commercially available kit, where isolated mRNA from CTCs is transcribed into cDNA and can be amplified in a following multiplex-PCR for the transcripts of Epithelial cell adhesion molecule (EpCAM), Mucin 1, Cell Surface Associated (MUC-1) and HER-2. Sieuwerts et al. established a robust method to perform mRNA expression analysis of multiple genes by RT-qPCR on small numbers of CTCs enriched from whole blood by the CellSearch system. This method allows molecular characterization specific for as little as one CTCs, and can be used to expand the understanding of the biology of metastasis and, potentially, to improve patient management (Sieuwerts et al., 2009).

2.3. *Liquid bead array*

Liquid bead array is a combination of multiplex RT-PCR with a flow-cytometry based approach that enables the simultaneous study of multiple gene expression in CTCs, while saving precious sample and reducing the costs and time of analysis. This methodology is based on the isolation of mRNA from immunomagnetically enriched CTCs followed by multiplex PCR for the selected gene transcripts. All transcripts are biotinylated through PCR, and biotinylated amplicons are then hybridized to gene-specific capture probes, that are coated on the surface of fluorescent microspheres. After hybridization, biotinylated PCR amplicons are incubated with streptavidin-phycoerythrin and are then quantified by Luminex flow cytometry. The expression of six gene-transcripts in CTCs has been measured simultaneously and this approach forms an efficient basis for a multiplex approach to study the expression of up to 100 genes and splice variants as well in CTCs (Markou et al., 2011; Parisi et al., 2012).

A combination of positive immunomagnetic selection of CTCs, using EpCAM and MUC-1 beads (Adnagen, Qiagen Hannover) and the WG-DASL HT assay was recently used for

gene expression profiling of more than 29,000 genes in a low number of cells (5–200). This protocol has been evaluated by spiking experiments of a known number of cancer cell lines in peripheral blood, and shows promise as technically reliable approach to obtain gene expression profiles from a small number (at least 25) of isolated CTCs (Fina et al., 2015).

3. Gene expression profiling of CTCs

3.1. *Breast cancer*

Through numerous clinical studies it is now evident that detection of CTCs in patients diagnosed with breast cancer both at the metastatic or early stage indicates poor prognosis and worse overall survival (Bidard et al., 2012; Zhao et al., 2011). Moreover, numerous clinical trials evaluate CTCs as markers for early prediction of treatment efficacy (Bidard et al., 2012).

3.1.1. *Early breast cancer*

One of the most studied genes studied at the gene expression level in CTCs is *CK-19*; the expression of this gene has been successfully used as a molecular marker for the detection of tumor cells in the bone marrow, lymph nodes and peripheral blood by RT-PCR. An RT-qPCR assay for *CK19*-mRNA was developed and validated more than ten years ago (Stathopoulou et al., 2003). It is important to notice that by using this RT-qPCR assay for *CK-19*, differences in the tumor load of CTCs (expressed as *CK-19* mRNA transcripts) before and after therapy were shown for the first time in early and metastatic breast cancer patients (Stathopoulou et al., 2003). A further optimization of this assay, based on a novel *in-silico* design of primer sequences, was important to avoid amplification of known pseudogenes for *CK-19*, while discarding the first milliliters of blood after venipuncture, contamination by skin epithelial cells, leading to false positive results was avoided (Stathopoulou et al., 2006). By using this quantitative RT-qPCR assay, a variety of clinical studies have shown that the detection of *CK-19* mRNA positive cells in the peripheral blood of patients with operable breast cancer before, during and after adjuvant treatment is an independent prognostic factor associated with an increased risk of disease relapse and shorter survival (Xenidis et al., 2009, 2007, 2013, 2006; Androulakis et al., 2012; Daskalaki et al., 2009; Ignatiadis et al., 2007, 2008). Moreover, by using this assay it was shown that persistent detection of CTCs during the first five years of follow-up was associated with an increased risk of late disease relapse and death in patients with operable breast cancer and indicates the presence of chemo-and hormonotherapy-resistant residual disease (Saloustros et al., 2011).

The detection rate of a three-marker-positive RT-PCR assay (*CK-19*, human mammaglobin (hMAM) and carcinoembryonic antigen (CEA)) in the blood of patients with early breast cancer was 54.0%, significantly higher than in patients with benign breast disease and healthy blood donors, while after three years of follow-up, detection of three-marker-positive CTCs was significantly associated with locoregional recurrence and/or distant metastasis (Chen et al., 2010). Another multimarker real-time quantitative PCR platform, with a specificity of 95%, detected CTCs in peripheral blood of 56% of patients with operable breast cancer. When this assay was used for the follow-up of 72 patients who received systemic adjuvant chemotherapy, it was shown that systemic adjuvant chemotherapy had a significant impact on CTCs status (Chong et al., 2012). Another PCR-based detection of CTCs was based on the amplification of six genes that were over-expressed in blood samples from 81% of patients with advanced and 29% of patients with primary breast cancer. These genes were selected out of 380 genes that were initially screened (Obermayr et al., 2010).

3.1.2. *Metastatic breast cancer (MBC)*

Gene expression assays, based on RT-PCR have been widely used to characterize CTCs and evaluate their clinical significance in metastatic breast cancer (MBC). In MBC, the median PFS and the OS were significantly shorter in patients with detectable CK-19mRNA-positive CTCs compared to patients without detectable CTCs (Androulakis et al., 2012). The presence of baseline CK-19mRNA-positive CTCs in patients with metastatic breast cancer was associated with poor prognosis while a decrease in mammaglobin positive mRNA CTCs may help predict response to therapy of MBC patients (Reinholz et al., 2011). Triple-marker-positive (EpCAM, CK19, and mammaglobin) CTCs, as evaluated by RT-PCR, were detected in the great majority (87.8%) of patients with metastatic breast cancer, and when compared to single-marker detection, the triple combined marker detection exhibited significantly higher rate. After 2 years of follow-up, the presence of CTCs using this triple-marker RT-PCR assay was an independent risk factor for reduced PFS and OS (Zhao et al., 2013). Gene expression profiling of isolated CTCs by RT-qPCR may provide a means for molecular characterization of selected tumor targets (Bao et al., 2013). Recently, quantification of mRNA expression by RT-qPCR in CTCs isolated from 197 patients with MBC was evaluated in relation to time-to-treatment failure (TTF) and has led to the generation of a prognostic CTCs 16-gene profile that could identify patients with a better outcome. However, validation of this profile in an independent cohort is needed before clinical application (Mostert et al., 2015a).

3.1.3. *Gene expression studies for the molecular characterization of CTCs in breast cancer*

In early breast cancer, the development of molecular profiling techniques has added prognostic and predictive information to conventional breast cancer biomarkers, such as estrogen receptor (ER) progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), while in the metastatic setting, recent findings are very promising and point towards a personalized management of cancer (Toss and Cristofanilli, 2015). From this point of view, the identification of the expression of specific molecular targets on CTCs is of great importance.

3.1.3.1. *EMT*. Epithelial-mesenchymal transition (EMT) is an essential process in the metastatic cascade (Lim and Thiery, 2012). The number of data demonstrating directly the existence of the EMT process in CTCs is constantly increasing (Bednarz-Knoll et al., 2012). Another study, using the commercially available Adnatest™ (Qiagen, Hannover, Germany) assay

that is based on multiplex RT-PCR evaluated the expression of EMT markers and ALDH1 in CTCs from primary breast cancer patients. This study has shown that a subset of primary breast cancer patients shows EMT and stem cell characteristics and that the currently used detection methods for CTCs are not efficient to identify a subtype of CTCs which underwent EMT (Kasimir-Bauer et al., 2012). Very recently it was shown by using RT-qPCR that the expression of the EMT-related biomarker plastin3 (PLS3) in CTCs of patients with stage I-III cancer, (particularly in patients with luminal-type and triple-negative-type tumors) could be a novel prognostic factor since PLS3-positive patients showed significantly poorer OS and DFS (Ueo et al., 2015).

3.1.3.2. *HER2*. There is a growing body of evidence that in breast cancer patients the HER-2 status in primary tumors can be very different than in CTCs, and may even change over time, especially during disease recurrence or progression (Sieuwerts et al., 2011; Ignatiadis et al., 2011; Fehm et al., 2009, 2010; Tewes et al., 2009; Punnoose et al., 2010). Thus, re-evaluation of HER-2 status by molecular characterization of CTCs is a strategy with potential clinical application. This was clearly shown in a recent randomized clinical study that evaluated the effect of trastuzumab on women with HER-2 negative early breast cancer but detectable CK-19 mRNA positive CTCs before and after adjuvant chemotherapy; these women were randomized to receive either trastuzumab or observation and it was shown that the administration of trastuzumab can eliminate chemotherapy-resistant CK-19 positive CTCs and reduce the risk of disease recurrence (Georgoulias et al., 2012).

3.1.3.3. *Estrogen (ER) and progesterone receptor (PR) expression*. The expression of breast cancer predictive markers such as estrogen (ER) and progesterone receptor (PR) can change during tumor evolution over time. Therefore, reassessment of these markers at the time of disease progression on CTCs might help to optimize treatment decisions, since classic biopsies from metastatic sites are difficult to obtain, especially for repeated analysis. It has already been reported that the expression profile of ER/PR/HER2 between CTCs and the primary tumor is different and should be re-evaluated (Fehm et al., 2009). When the ER/PR expression on CTCs was compared to the hormone receptor status expression profile of the primary tumor, it was found that most of the CTCs were ER/PR-negative despite the presence of an ER/PR-positive primary tumor (Aktas et al., 2011a).

Overall molecular assays are giving important information for the molecular characterization of CTCs in breast cancer and many clinical studies support their potential utility in breast cancer.

3.2. *Colorectal cancer*

Gene expression profiling of the tumor-associated genes CK19, MUC1, EPCAM, CEACAM5 and BIRC5, by RT-qPCR suggest that in addition to the current prognostic factors, gene expression analysis of CTCs represents a potential complementary tool for prediction of colorectal cancer patients' outcome (de Albuquerque et al., 2012a). RT-qPCR gene expression analysis data of CEA CK19, CK20 and/or CD133 (CEA/CK/CD133) in CTCs of CRC patients with Dukes' stage B and C suggest that detecting CEA/CK/CD133 mRNA in tumor drainage blood by RT-qPCR could have a prognostic value in CRC patients with Dukes' stage B and C (Shimada et al., 2012).

Plastin-3 (PLS3) was recently proposed as a novel CTCs marker for metastatic CRC cells that possesses significant prognostic value. Yokobori et al. found that PLS-3 was expressed in metastatic CRC cells but not in normal circulation and that this molecular marker was expressed in EMT-induced CTCs in peripheral blood from patients with CRC with distant metastasis. This study has shown that the association between PLS3-positive CTCs and prognosis was particularly strong in patients with Dukes B and Dukes C (Yokobori et al., 2013). Using RT-qPCR Iinuma H et al., studied the expression of carcinoembryonic antigen (CEA), CK19, CK20, and/or CD133 (CEA/CK/CD133) in CTCs of 735 patients with CRC, and reported that OS and DFS of patients with Dukes' stage B or C cancer who were positive for CEA/CK/CD133 were significantly worse than those of patients who were negative for these markers (Iinuma et al., 2011). In clinical CRC cases, high expression of PLS3 in CTCs of tumor drainage venous blood (TDB) as well as peripheral blood was established as an independent prognostic factor of OS. PLS3 induced the EMT via transforming growth factor (TGF)-β signaling and resulted in the acquisition of invasive ability in CRC cells (Sugimachi et al., 2013).

The detection of CTCs might identify CRC patients at high risk of dying of disease recurrence after apparently radical liver surgery. By RT-qPCR Pilati et al. studied the expression of a panel of cancer-related genes, and reported that CD133-positive CTCs may represent a suitable prognostic marker to stratify the risk of patients who undergo liver resection for CRC metastasis (Pilati et al., 2012). Very recently, Mostert and colleagues using RT-qPCR, aimed to identify colorectal tumor-specific gene expression levels in the blood of patients with and without detectable CTCs according to CellSearch criteria. According to their results, 34 CTC-specific mRNAs were higher expressed in patients with $\geq$3 CTCs/Ml according to the CEllSearch, while a subgroup of patients without detectable CTCs according to CellSearch criteria were found to bear circulating tumor load, which may have clinical consequences (Mostert et al., 2015b).

Overall molecular assays are giving important information for the molecular characterization of CTCs in colorectal cancer however their potential clinical utility remains to be proven.

3.3. *Prostate cancer*

Molecular determinants can be identified and characterized in CTCs of prostate cancer patients as potential predictive biomarkers of tumor sensitivity to a therapeutic modality (Danila et al., 2011a). Recently the molecular characterization of CTCs in prostate cancer patients has proven to be of major clinical significance, and molecular assays based on RT-PCR very powerful to predict which patients will respond to specific treatments. Especially metastatic castration-resistant prostate cancer (mCRPC) that currently benefits from many treatment options still remains lethal in the vast majority of

patients. The role of CTCs in this disease that continues to evolve over time, is now emerging as a biological window to diverse resistance mechanisms.

3.3.1. AR-V7

AR-V7 is an androgen-receptor isoform, encoded by splice variant 7 that lacks the ligand-binding domain, which is the target of enzalutamide and abiraterone, but remains constitutively active as a transcription factor. The recent discovery of alternate splice variants of the androgen receptor (AR) is one potential mechanism of escape in mCRPC. AR-V7 appears to be the most relevant AR splice variant, and early clinical data suggest that it is a negative prognostic marker in mCRPC. AR-V7 is a dynamic marker whose status may change across time and depending on selective pressures induced by different therapies. Thus following alterations of AR-V7 expression in CTCs is of major clinical importance (Maughan and Antonarakis, 2015). Antonarakis et al. were the first to use RT-qPCR to evaluate AR-V7 expression in CTCs from prospectively enrolled patients with metastatic castration-resistant prostate cancer (CRPC) who were initiating treatment with either enzalutamide or abiraterone. They report that detection of AR-V7 in CTCs from patients with CRPC is associated with resistance to enzalutamide and abiraterone (Antonarakis et al., 2014). Based on these findings, many clinical studies are now evaluating AR-V7 as a treatment selection biomarker in CRPC. According to another recent study, detection of AR-V7 in CTCs from men with metastatic CRPC is not associated with primary resistance to taxane chemotherapy; iIn AR-V7-positive men, taxanes appear to be more efficacious than enzalutamide or abiraterone therapy, whereas in AR-V7-negative men, taxanes and enzalutamide or abiraterone may have comparable efficacy (Antonarakis et al., 2015). The association between AR-V7 expression in CTCs and resistance to cabazitaxel was also very recently investigated in mCRPC patients. The expression of AR-V7 was assessed by RT-qPCR in CTCs, and it was found that cabazitaxel was effective independently of the presence of AR-V7 in CTCs (Onstenk et al., 2015). Very recently, single-cell RNA-sequencing (RNA-Seq) profiles of 77 intact CTCs isolated from 13 patients has revealed that single CTCs from each individual display considerable heterogeneity, including expression of AR gene mutations and splicing variants. This heterogeneity in signaling pathways could possibly contribute to treatment failure (Miyamoto et al., 2015).

3.3.2. PSA, PSMA

Detection of CTCs based on RT-qPCR for prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) mRNA in patients' pre- and post-radical prostatectomy was shown to predict the probability of recurrence-free survival (Yates et al., 2012).

3.3.3. TMPRSS2-ERG status

Abiraterone acetate is an androgen biosynthesis inhibitor shown to prolong life in patients with CRPC already treated with chemotherapy. This treatment results in dramatic declines in PSA levels in some patients and no declines in others, suggesting the presence of molecular determinants of sensitivity in tumors. Danila et al., studied the role of transmembrane protease, serine 2 (TMPRSS2)-v-ets erythroblastosis virus E26 oncogene homolog (ERG) fusion, an androgen-dependent growth factor, in CTCs as a biomarker of sensitivity to abiraterone acetate. Using a sensitive, analytically valid RT-PCR they have shown that TMPRSS2-ERG status did not predict a decline in PSA or other clinical outcomes. This finding demonstrates the role of CTCs as surrogate tissue that can be obtained in a routine practice setting (Danila et al., 2011b). Attard et al., have also used RT-qPCR and came to the conclusion that there is a significant association between ERG rearrangements in therapy-naive tumors, CRPCs, and CTCs and magnitude of prostate-specific antigen decline in CRPC patients treated with abiraterone acetate (Attard et al., 2009).

Overall molecular assays are giving important information for the molecular characterization of CTCs in prostate cancer and an increasing number of clinical studies support their potential clinical utility in prostate cancer.

3.4. *Pancreatic cancer*

Up to now there are just a few papers published on gene expression in CTCs of patients with pancreatic cancer. Recently, the Haber group compared genome-wide expression profiles of CTCs with matched primary tumors in the genetically engineered LSL-$Kras^{G12D}$, $Trp53^{flox/flox}$ or + Pdx1-Cre (KPC) mouse model. They isolated individual CTCs using epitope-independent microfluidic capture, followed by single-cell RNA sequencing and report that human pancreatic CTCs exhibit a very high expression of stromal-derived extracellular matrix (ECM) proteins, whose knockdown in cancer cells suppresses cell migration and invasiveness (Ting et al., 2014). The same group has recently identified WNT2 as a candidate gene enriched in CTCs of pancreatic cancer patients. They report that expression of WNT2 in pancreatic cancer cells suppresses anoikis, enhances anchorage-independent sphere formation, and increases metastatic propensity in vivo (Yu et al., 2012).

A multimarker RT-PCR assay was used to evaluate CK-19, MUC1, EpCAM, CEACAM5 and BIRC5 expression in CTCs of pancreatic cancer patients. CTCs were detected in 47.1% and patients who had at least one detectable tumor-associated transcript showed shorter median progression-free survival compared with patients who were CTCs negative (de Albuquerque et al., 2012b). Sergeant et al. prospectively studied the value of a real-time RT-PCR assay for EpCAM detection in the peripheral blood and peritoneal cavity of patients undergoing pancreatectomy for pancreatic ductal adenocarcinoma. Despite a significant increase in EpCAM counts in postoperative blood and peritoneal lavage fluid this was not associated with worse prognosis after pancreatectomy for pancreatic ductal adenocarcinoma (Sergeant et al., 2011). Very recently the role of EMT in pancreatic ductal adenocarcinoma (PDAC) was studied by generating mouse models of PDAC with deletion of Snail or Twist, two key transcription factors responsible for EMT. This study highlights the importance of combining EMT inhibition with chemotherapy for the treatment of pancreatic cancer (Zheng et al., 2015).

Overall molecular assays are very promising for the molecular characterization of CTCs in pancreatic cancer; their clinical utility remains to be proven.

3.5. *Ovarian cancer*

Up to now there are just a few papers published on gene expression in CTCs of patients with ovarian cancer. By using the AdnaTest™ (Qiagen, Hannover, Germany) Aktas et al. detected CTCs in the blood of 122 ovarian cancer patients at primary diagnosis and/or after platinum-based chemotherapy. The assay was based on immunomagnetic enrichment, targeting common antigens on epithelial gynecological cancers, followed by multiplex RT-PCR for EpCAM, MUC-1, and HER-2 transcripts and CA-125 that was assessed in an additional single-plex RT-PCR,. According to the results presented in this study, CTCs positivity in ovarian cancer patients significantly correlated with shorter OS before surgery and after chemotherapy (Aktas et al., 2011b). Recently the same group has shown that expression of the excision repair cross-complementation group 1 (ERCC1) gene in the form of the ERCC1 transcript in CTCs can serve as a blood-based diagnostic biomarker for predicting platinum resistance at primary diagnosis of ovarian cancer (Kuhlmann et al., 2014).

Another group that works since many years on the molecular characterization of CTCs in ovarian cancer has recently shown that molecular characterization of CTCs is superior to a mere CTCs enumeration in epithelial ovarian cancer. Gene expression of novel markers as derived from microarray analysis data was analyzed using RT-qPCR in blood samples taken from healthy females (N = 39) and from epithelial ovarian cancer patients (N = 216) before primary treatment and six months after adjuvant chemotherapy. It was found that cyclophilin C gene (PPIC) was overexpressed in CTCs and that PPIC positive CTCs during follow-up were significantly more often detected in the platinum resistant than in the platinum sensitive patient group, and indicated poor outcome independent from classical prognostic parameters (Obermayr et al., 2013).

Overall molecular assays are giving important information for the molecular characterization of CTCs in ovarian cancer; their clinical utility remains to be proven in ovarian cancer.

4. DNA methylation analyses of CTCs

It was only very recently that the epigenetic state of CTCs has started to be studied. The epigenetic silencing of key tumor suppressors and metastasis suppressors was detected in CTCs for the first time by using Methylation specific PCR (MSP) for cystatin M (*CST6*), Breast Cancer Metastasis Suppressor-1 (*BRMS1*) and SRY-box containing gene 17 (*SOX17*) gene promoters (Chimonidou et al., 2011). These genes were carefully selected to be studied in CTCs at the DNA methylation level, since it was already known that their expression at the protein level affects hallmark properties of tumor cells, including growth and proliferation, invasiveness, epithelial phenotype and stemness.

4.1. *SOX17*

SOX17, a transcription factor, is considered as an antagonist to canonical Wnt/β-catenin signaling in several types of malignant tumors. Interestingly, in CTCs, *SOX17* was found to be highly methylated both in patients with early and patients with metastatic breast cancer (Obermayr et al., 2013). *SOX17* promoter was also found to be highly methylated in primary breast tumors, in CTCs isolated both from early and metastasis verified breast cancer patients, and in corresponding cell free DNA (cfDNA) samples (Chimonidou et al., 2013a). A key note of these findings is that it has been shown for the first time that *SOX17* promoter methylation in CTCs and in matched cfDNA is highly correlated. This finding shows towards a direct connection between the presence of CTCs and cfDNA in operable breast cancer patients, after surgical removal of the primary tumor. It is very interesting to note that according to recent findings, SOX17 is involved in the regulation of EMT, stemness and metastasis (Li et al., 2015). Moreover the information on DNA methylation of *SOX17* in CTCs can be extremely important if we take into account that the expression of SOX17 is negatively controlling the WNT pathway, while methylation of *SOX17* promoter is silencing this gene. Combining this information with the recent finding that the expression of WNT2 in pancreatic cancer cells suppresses anoikis, enhances anchorage-independent sphere formation, and increases metastatic propensity in vivo can lead to interesting conclusions (Yu et al., 2012).

4.2. *BRMS1*

Breast cancer metastasis suppressor 1 (BRMS1), a known suppressor of metastasis, is a predominantly nuclear protein that differentially regulates expression of multiple genes, leading to suppression of metastasis without blocking orthotopic tumor growth (Phadke et al., 2008). This gene is significantly downregulated in some breast tumors, especially in metastatic disease, by epigenetic silencing (Metge et al., 2008). BRMS1 coordinately regulates expression of multiple metastasis-associated miRNAs (Hurst et al., 2009), while its expression in metastatic human breast cancer cells leads to selective repression of epidermal growth factor receptor and downstream AKT signaling (Vaidya et al., 2008). In CTCs of patients with operable breast cancer, promoter methylation of BRMS1 was observed in 32.1%, while in CTCs of patients with verified metastasis, promoter methylation of BRMS1 was observed in 44.4% (Obermayr et al., 2013). BRMS1 promoter was found to be highly methylated in CTC, while immunofluorescence studies in the same cytospins have shown that it was down regulated at the protein level in CTC as well (Chimonidou et al., 2013b). Moreover, we have recently shown that methylation of BRMS1 promoter in circulating tumor DNA isolated from plasma of NSCLC patients provides important prognostic information and merits to be further evaluated as a circulating tumor biomarker (Balgkouranidou et al., 2014).

4.3. *CST6*

Cystatin M or E/M (encoded by the CST6 gene) is an endogenous inhibitor of cathepsins B and L (Mohamed and Sloane, 2006) that was first identified by differential display of mRNAs as being markedly down-regulated in metastatic breast cancer (Sotiropoulou et al., 1997) and mapped to chromosomal locus 11q13 (Stenman et al., 1997). DNA hypermethylation, in this region impairs transcription and leads to loss of cystatin M

expression in cancer (Shridhar & et al., 2004; Ai et al., 2006; Pulukuri & et al., 2009; Rivenbark et al., 2006). Cystatin M has generated much recent interest and is postulated to be a tumor suppressor in breast cancer (Ai et al., 2006). We demonstrated already in 2009 that CST6 promoter methylation provides important prognostic information when evaluated in primary tumor tissues of patients with operable breast cancer (Kioulafa et al., 2009). Our group has also demonstrated that CST6 promoter is highly methylated in circulating tumor DNA of breast cancer patients, but not in healthy individuals (Chimonidou et al., 2013c). In CTCs of patients with operable breast cancer, promoter methylation of CST6 was observed in 17.9%, of patients. In CTCs of patients with verified metastasis, promoter methylation of CST6 was observed in 37.0%, of patients, while in healthy individuals, promoter methylation of CST6 was observed in only 4.3% (Chimonidou et al., 2011).

According to a recent study, exploring the feasibility of invasive CTCs epigenomic analysis in a very limited number of patients with mCRP, the invasive CTCs methylation profile highly resembled mCRPC primary tumors (Friedlander et al., 2014). However, the detection and molecular characterization of CTCs at the DNA methylation level is still very limited but the potential of this field to elucidate mechanisms of metastasis and to develop novel tumor biomarkers is challenging (Pixberg et al., 2015).

5. Conclusions and future challenges

CTC analysis is very challenging and demanding, since CTCs are not only rare, but highly heterogeneous as well, even within the same patient. An additional analytical and technical challenge is the fact that the amount of available CTC sample for analysis is very limited, while the number of interesting targets to be evaluated on CTCs is constantly increasing. Molecular assays and especially multiplex RT-PCR offer a unique advantage for the detection and molecular characterization of CTCs. The main advantages of molecular assays for CTC detection and molecular characterization are their high sensitivity and the fact that they can be *a-priori* designed *in silico*, quantitative, high throughput and easy to perform, while they usually require a very small sample quantity for analysis. Analytical validation of molecular assays before their application in precious CTC samples is of vital importance. Molecular assays are low cost, and can be subjected to a quantifiable quality control system. So far, their clinical applications in many types of cancer have shown that they can give clinically relevant information. In addition they have the potential to identify specific therapy-related molecular targets on CTCs such as gene expression, chromosomal translocations, or gene mutations expressed on CTCs. This information could be very important, especially in early steps of the disease to choose for the correct treatment and moreover explain resistance to established therapies.

The molecular characterization of CTCs at the single cell level represents a major challenge nowadays, since through this way we look forward to understand the importance of CTC heterogeneity in the same patient and the potential role of individual CTCs with specific characteristics in cancer dissemination, metastasis, and response to specific treatments. Recent technologies that focus on the accurate quantification of gene expression at the single cell level hold great promise to reveal the importance of CTCs variability in the clinical setting. Very recently, high-throughput methods for single-cell RNA-seq have been introduced that vary in coverage, sensitivity and multiplexing ability (Picelli et al., 2014). Single-cell RNA-sequencing (RNA-Seq) profiles of intact CTCs isolated from prostate cancer patients has already revealed heterogeneity in signaling pathways that could contribute to treatment failure (Miyamoto et al., 2015). Another recent study has clearly shown for the first time the potential of comprehensive genomic profiling of CTCs using array-comparative genomic hybridization (aCGH) and next-generation sequencing and paved the way to monitor tumor genomes that are prone to change during progression, treatment, and relapse (Heitzer et al., 2013). Technological advances that enable today single-cell CTC analysis may help elucidate tumor heterogeneity at the CTC level (Salvianti et al., 2016), but there are still challenges to be met such as single cell RT-qPCR of stained CTCs.

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