Real-time RT-PCR systems for CTC detection from blood samples of breast cancer and gynaecological tumour patients (Review)

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Abstract. Cells, which detach from a primary epithelial tumour and migrate through lymphatic vessels and blood stream are called 'circulating tumour cells'. These cells are considered to be the main root of remote metastasis and are correlated to a worse prognosis concerning progression-free and overall survival of the patients. Therefore, the detection of the minimal residual disease is of great importance regarding therapeutic decisions. Many different detection strategies are already available, but only one method, the CellSearch® system, reached FDA approval. The present review focusses on the detection of circulating tumour cells by means of real-time PCR, a highly sensitive method based on differences in gene expression between normal and malignant cells. Strategies for an enrichment of tumour cells are mentioned, as well as a large panel of potential marker genes. Drawbacks and advantages of the technique are elucidated, whereas, the greatest advantage might be, that by selection of appropriate marker genes, also tumour cells, which have already undergone epithelial to mesenchymal transition can be detected. Finally, the application of real-time PCR in different gynaecological malignancies is described, with breast cancer being the most studied cancer entity.

Contents

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1. Introduction

Gynaecological malignancies. A large number of different gynaecological malignancies affect women worldwide. The most common ones are cervical, ovarian, uterine, vaginal and endometrial carcinoma, breast cancer also belongs to this group but is not exclusively limited to women. All of these diseases are rather different in occurrence, screening possibilities and therapeutical concepts, but have in common, that still a large number of women are newly affected with one of these malignancies every year. Although death rates were decreasing in recent years, still many women die from their disease or the additional effects. The treatment strategies are mostly limited to surgery, radiation therapy, chemotherapy or hormonal therapy, but detection and combating of minimal residual disease could possibly improve death statistics greatly, as these circulating or disseminated tumour cells in the peripheral blood or bone marrow are considered as the origin of distant metastases.

General facts on circulating tumour cells (CTCs). Circulating tumour cells (CTCs) were first described in 1869 by Thomas Asworth, who found cells with the morphology of tumour cells in the blood of a person, who had died from metastatic cancer (1). Already 20 years later the 'seed and soil' hypothesis was drawn by Paget (2) and revised in 2003 by Fidler (3). It states, that for the formation of remote metastasis detached tumour cells on one hand and a certain organic background on the other hand is necessary. CTCs are rare events in the blood, following a Poisson distribution (4). Approximately 10^6 tumour cells enter circulation every day, but 85% disappear within five min (5), so that only one CTC can be detected in 10^5-10^7 mononuclear cells per ml blood (6). Only 2.5% of these CTCs are able to form micrometastases and 0.01% form macroscopic metastases (5), and CTCs are rather rare events in healthy persons (7,8). The release of CTCs is regarded as one of the early steps during tumour formation (9-11). Patients (40%), who still have tumour-free lymph nodes, already have CTCs in their blood stream (12), so that the measurement of CTCs is an important feature in tumour diagnostic. Therefore, the detection of CTCs is already included in international tumour staging systems (13,14). They can also be helpful in predicting disease progression and in monitoring treatment efficiency (15). Metastatic cells often have different features than the primary tumour (15-18). They are frequently

triple-negative for their hormone-receptor and Her2 status, which is linked to a more aggressive phenotype (19,20) and a lack of Ki-67 expression, which contributes to resistance to chemotherapy (21). Therefore, an explicit characterization of CTCs (22,23) is important for further application of clinical treatment.

When CTCs access bone marrow, they are called 'disseminated tumour cells' (DTCs) and they can reside there for many years, creating a kind of 'tumour reservoir' (24), which can give rise to metastasis at a later time (25). CTCs are highly heterogeneous (26) and in many cases show up with a triple-negative phenotype, concerning hormone and Her2-receptor status (16).

The limitation for CTC detection is the amount of blood available, so mostly an enrichment step is prepended in most methods (20).

CTCs and disease prognosis. The occurrence of metastasis causes an adverse prognosis in cancer patients (27,28). CTCs do not only give rise to metastasis in distant organs, but also self-seed back to their original organs (29). The detection of CTCs in patients with metastatic breast cancer predicts a worse disease-free survival and worse overall survival (DFS and OAS) (30). The effect of the occurrence of CTCs in primary breast cancer was summarized by Saloustros and Mavroudis (31). It was analysed by the Light Cycler™ method (32), with cytokeratin 19 (CK19) used as a marker gene (33). The occurrence of CTCs before administration of chemotherapy predicts an early metastatic relapse and disease-related death (34,35). The persistence of CTCs after adjuvant chemotherapy also gives a hint towards decreased DFS and OAS. Therefore, occurrence of CTCs before and after chemotherapy are independent prognostic factors for worse clinical outcome (36). This prognostic value of CTC occurrence is even increased in different molecular subtypes of breast cancer, for example in estrogen-receptor (ER) negative type (37). Furthermore, the prognostic value of CTCs was analysed after hormone therapy and in follow-up. A persistence of CTCs after tamoxifen-treatment of hormone-receptor positive breast cancer is predictive for short DFS/OAS (38), as is the case for CTCs which can be found month and years after finalization of therapy (39). Thereby CTCs seem to have a significant clinical utility in cancer treatment.

2. CTC detection

CTC enrichment and detection methods. The detection of CTCs from peripheral blood is easier in handling and less exhausting for the patients, and is, therefore, the method of choice. The drawback of this methodology is, that the number of CTCs is rather small in relation to the surrounding blood cells, especially in non-metastatic cancer forms (40). Therefore, most methods implicate a CTC-enrichment (positive selection) or lymphocyte depletion (negative selection) procedure before starting the real CTC detection. Apart from the GILUPI® nanodetector, which should be able to isolate more tumour cells than the Veridex™-system (41), but is not yet established in the clinical routine, there are two main techniques of CTC enrichment: density gradient centrifugation either by Ficoll-Hypaque or by OncoQuick® or an immunomagnetic enrichment of tumour cells by cell surface antigens (42). The drawback of immunomagnetic enrichment of tumour cells is that CTCs are highly heterogeneous in their antigen expression (43-45), so that methods using EpCAM as an enrichment marker miss the population of EpCAM-negative CTCs (45). It would be more reasonable, to use tumour specific antigens for enrichment, for example CD176 (46). Furthermore, CTCs can be isolated by size (ISET). A great advantage of this technique is, that cells are not modified during enrichment, so they can subsequently be used for FISH or PCR characterization assays (47). The RosetteSep™ system offers a negative selection of tumour cells by crosslinking unwanted blood cells to erythrocytes, simplifying their removal during density gradient centrifugation (48,49).

Many CTC detection methods combine a CTC enrichment step with further molecular analysis of tumour cells. The most important methods therefore should be summarized in the following: The 'gold standard' in CTC detection is still the CellSearch® system (26), which is approved by the FDA (Food and Drug Administration) and has been validated by large clinical testings (50). The system first uses an immunomagnetic enrichment based on EpCAM [meaning that EpCAM negative cells are not detected by this system, which is one of the great drawbacks of this method; (45)], secondly the isolated cells are subsequently labelled fluorescently for DAPI, CD45 and CK19, so that CTCs could be undoubtedly detected (7,51,52). The CTC-results of the CellSearch® system gives a prognostic patient information in respect to progression-free survival and overall survival (PFS and OAS, respectively (15). Already one circulating tumour cell in early breast cancer is a marker for a poor disease-free and overall survival, five or more CTCs found in a blood volume of 7.5 ml are a marker for worse prognosis (53). Even more CTCs in metastatic breast cancer are a sign of impending progression of the disease (54-57). An interesting option for the future could be to combine the CellSearch® system with ISET, as this method finds more CTC clusters, which develop during tumour cell division and could be important in metastatic processes (58). Apart from this method, there are protein-based strategies for CTC detection, like the EPISPOT-assay, which detects tumour cell specific proteins (59), so that only viable cells are found (26,60-62). By detection of cytokeratin 19 protein via EPISPOT-assay an active subset of breast cancer cells with metastatic properties can be detected (63). A detection of CTCs can be carried out by fluorescence *in situ* hybridization (FISH), which detects chromosome aberrations in the tumour cells (64,65), or miRNA-profiling, as an altered miRNA expression profiling gives hints towards initiation and the progression of cancer (66). Also a detection of CTCs by microdevices, so-called 'CTC-chips' (59,67), which capture EpCAM-expressing cells (68,69) or microarrays can be done. The advantage of the microarray technology is, that a large number of genes can be analysed simultaneously, but a huge amount of RNA (1-2 μg) is needed (70). This drawback is overcome by Pico Profiling, which does an expression profiling from as little as 10 tumour cells. Recently, also Raman spectroscopy (SERS) was used for circulating tumour cell detection (71). Another system for CTC detection is the so-called Ariol system, which is based on image capturing. Three fluorescent images of cell surface antigens like CK or EpCAM and one brightfield image of one

cell are taken together for detection of tumour cells (72). Flow cytometry can also be used for CTC detection, with the advantage, that multiple cell parameters, such as DNA-content, cell size and cellular markers can be analysed simultaneously and has thereby advanced to be a specific method to monitor breast cancer patients receiving chemotherapy (73).

Additionally to all these methods mentioned above there are mRNA-based methods for CTC detection, which are the main focus of the present review and will be described in detail in the following sections.

RNA-based methods for CTC detection. The detection of viable CTCs by a detection of epithelial or tumour specific mRNAs by qRT-PCR is one of the most widely used techniques in the CTC-research field (74-78). QRT-PCR is the abbreviation for quantitative (real-time) reverse transcription PCR (79), which means that RNA is isolated from the tissue of interest (blood or fraction of a blood sample), is converted to cDNA by a reverse transcriptase reaction, which is then used in a real-time PCR reaction. For this quantification of RNA targets (80), which over the last years has turned into one of the mainstream research technologies (81), due to the high sensitivity of detection of small RNA amounts, even in a background of high total mRNA (70). A suitable detection chemistry, an instrument for monitoring of gene expression software, equipped with an adequate software are necessary (82). Thereby the time-point, when the PCR-product is first detected during cycling is recorded and can be translated into gene expression values. There are two different methods for quantification of PCR products: absolute and relative quantification (80). For absolute quantification it is necessary to generate a standard curve of known target numbers first, to later correlate real-time PCR recordings to a transcript number (83). In a relative quantification study internal reference genes, mostly housekeeping genes like GAPDH, 18S, RPLP0 or B2M (84) are run simultaneously with the genes of interest, and their gene expression values are correlated to those of the reference genes (85). The disadvantage of relative quantification is, that even reference genes can vary in gene expression, resulting in a misleading quantification (86). There are also two different options in the usage of detection chemistry: These could be non-probe based (like SYBR-Green), which requires the generation of a melting curve after completion of real-time PCR reaction (87), to distinguish specific PCR products from unspecific ones. More specific is the use of probe-based detection chemistry, which uses target specific PCR-primers, which can additionally be labelled with different fluorophores, making multiplexing possible (88). A higher sensitivity in the detection of target gene expression can also be achieved by Nested PCR-approaches (89). The advantages of the real-time PCR assays are: DNA is amplified and analysed in only one experimental step, targets, which differ widely in their copy number can be analysed during the same reaction, a rather small inter-assay variation, the quantitative capacity of the method (90,91), and the possible suggestion of tumour spread by detection of tissue specific RNAs (92). There are also disadvantages, like the complicated handling of RNA samples due to the inherent presence of RNases, the use of oligo(dT)-primers, which on one hand contributes a high specificity to the reaction, but on the other hand requires the use of high quality RNA, the risk of a copurification of PCR inhibitors during the procedure, which could lower PCR efficiency (93,94) and the risk of false-positive results due to minimal contaminations (76,95,96). Furthermore, no morphological analysis of the detected tumour cells is possible (6) a heterogeneous expression of target genes might lead to a non-accurate enumeration of tumour cells, and many transcripts are expressed at low levels in normal blood cells (97,98), leading to false-positive results, which makes the selection of target genes a crucial point in this experimental approach. Therefore, a stringent quality control has to be applied during the whole procedure, including use of reference guidelines, positive and negative controls, optimal sample quality and primer selection, adequate instrument care, and analytical sensitivity and specificity (99-103). If all of these steps are conducted carefully, reliable quantitative data can be obtained, which could in term lead to a personalized medicine (97), and even further: a detection of CTCs in early stage cancer could influence the application of different treatment options (104,105).

A CTC-detection method assisted by real-time PCR is the AdnaTest™ (AdnaGen AG), in which tumour cells are first immunomagnetically enriched by epithelial and tumour specific antibodies and subsequently analysed by multiplex-qPCR (51). This combination of markers helps to detect even rare CTCs, and thereby it was found that a major proportion of CTCs show characteristics of epithelial-mesenchymal transition (EMT, which is discussed later on) and have stem cell properties (106).

RT-PCR marker genes for CTC detection. The main purpose of RT-qPCR is to amplify epithelial or cancer specific genes (107), but such marker genes are sometimes also expressed in blood cells (108), or lack sensitivity (109), so that only a few molecules can be used for detection (110) and the choice of marker genes is one of the crucial points in the PCR-process.

CK 19 is one of the target genes frequently used in real-rime PCR for the detection of circulating tumour cells in clinical studies (35,36,98,107,111-114). Silva *et al* (115) described also the use of EGFR VIII for the detection of CTCs, but the most widely described marker gene, especially in breast cancer analyses for RT-PCR is Mammaglobin A (hMAM) (116-118). The gene region of hMAM is frequently found to be amplified in breast cancer tissues (119). This expression is limited to the adult mammary gland (120) and related to mammary gland proliferation and differentiation (110,121-125), but is commonly absent in healthy breast tissue samples. The detection frequencies seem to vary (126-128), sensitivity of qPCR for detection of hMAM mRNA shows a broad range even in metastatic breast cancer (128-130), imposing doubt on the utility of this marker (118), but a combination of hMAM with survivin and hTERT increases the sensitivity of CTC detection (131), pointing towards the use of multimarker PCR (132). Furthermore, no correlation could be found between the expression of hMAM and nodal state, tumour size and grading (109,121,132). The problem of the application of hMAM as a RT-PCR marker gene is, that it is an epithelial gene, and its expression could be altered during the process of EMT leading to false-negative results.

Epithelial-mesenchymal transition. During the process of cell detachment from the primary tumour and the invasion of the blood vessel tumour cells often undergo the process of EMT, meaning that they lose their epithelial gene expression panels and cellular properties, like the ability of cell adhesion and the apical-basal cell polarity (70) and switch to a mesenchymal phenotype with increased invasiveness and resistance to apoptosis (61,70,133) and furthermore, increased resistance to anticancer therapies (134,135). The EMT dedifferentiation process is associated with an increased tumour cell aggressiveness (136). Patients who have CTCs without cytokeratin and other epithelial features but with mesenchymal markers have a worse prognosis for survival (137). The problem that the EMT-process bears for the detection of CTCs is, that such cells would escape to an enrichment via epithelial markers such as EpCAM (138), or cannot be detected by the use of epithelial marker genes.

Drawbacks and requirements of real-time PCR based tumour cell detection. The most serious drawbacks of the real-time PCR methodology for the detection of circulating tumour cells are, that, due to a large number of experimental steps and a certain variability therein, a comparison of different studies is difficult. Furthermore, there is a low reproducibility of the results obtained, caused by suboptimal standardization of the experimental processes. Additionally, frequently there are only small sample sizes available for analysis, so that a statistical evaluation of results could be difficult (111). Another disadvantage of the method is, that, by measurement of gene expression, CTC numbers present in a certain sample cannot be measured accurately, but can be characterized for a number of gene expression features (97). To overcome these drawbacks it is important to standardize first of all, the preanalytical phase, *i.e*. the sampling, then the isolation of the tumour cells as RNA from those cells, the inclusion of spiking experiments for quality control, and last but not least, the detection systems. Only then an intra- and inter-laboratory comparison of RT-qPCR studies would be possible (20).

3. Detection of CTCs in gynaecological malignancies

General facts. Detection and characterization of CTCs have recently become important issues in tumour diagnosis and management. As CTCs express a different gene panel than normal blood cells, a global gene expression profiling was carried out in order to distinguish normal donors from advanced cancer patients, and even to distinguish patients with different cancer entities (139). The challenge thereby is to differentiate CTCs from large numbers of surrounding lymphocytes with molecular techniques like multiplex real-time PCR (45). The group of De Albuquerque *et al* (140) developed such an assay based on immunomagnetic enrichment of tumour cells with subsequent real-time PCR analysis of the extracted cells. They were able to create a marker gene panel for the detection of CTCs from adenocarcinomas of 10 different organs. These different techniques for tumour cell detection gain in importance, as CTCs are more and more relevant not only for prognosis but also for therapy monitoring (141). However, it is becoming increasingly obvious, that CTC enrichment concepts based on EpCAM are no longer adequate for an extensive analysis of circulating tumour cells (142), as cells, which detach from a primary tumour undergo epithelial-mesenchymal transition (EMT), changing their gene and protein expression, escaping detection by most of the actually used methods. Furthermore, the EMT process is known to be extremely important for metastasis formation, so that especially tumour cells, which have undergone this process are important for clinical tumour management (143), creating the future need of new detection routines.

Recent developments for CTC detection in breast cancer. As breast cancer currently affects 6% of the female population (144), there is a huge medical research focus on this cancer entity. The real-time PCR methodology was widely used for CTC-detection from blood samples of breast cancer patients using marker genes such as CK19 (145). It was shown, that the occurrence of CK19-positive CTCs is correlated with the incidence of CK19-positive DTCs and is linked to a decreased survival of patients with early stage breast cancer. Cytokeratins in general were also, in another study, shown to be rather useful for CTC detection (146) especially in metastatic breast cancer patients. The gene for matrix metalloprotease was in contrast shown to be a useful marker for CTCs which already had undergone EMT (147). Another marker for detection of CTCs from breast cancer patient blood samples was h-MAM, which was demonstrated to be especially useful for evaluation of treatment efficacy and post-treatment monitoring of patients (148). As cancers often have a great heterogeneity in their gene expression many studies aim to find a suitable set of real-time PCR marker genes for the detection of CTCs. Xi *et al* (149) for example screened a set of 52 potential marker genes to find a set of three to eight marker genes for a number of cancer entities. De Albuquerque *et al* (140) found a set of marker genes, which could be helpful for the detection of CTCs from blood of metastatic breast cancer patients, Lasa *et al* (150) described an increase in CTC-detection rate by use of a marker set in comparison to the use of a single gene. A detection of two genes, the PTHRP and CK19 correlates furthermore with the incidence of remote metastasis (151), also giving hint to the use of a combination of marker genes for CTC detection and even more characterization.

Another important topic in CTC detection via real-time PCR is of course the quantification of tumour cells. Two studies were set up with cells from breast cancer cell lines added, in certain amounts, to blood samples of healthy donors, and processing these blood samples just like patient samples to real-time PCR. Both studies found, that cytokeratins are suitable PCR marker genes for CTC quantification from blood samples, promising new possibilities in CTC detection, treatment assessment and follow-up (152). The sensitivity of the CTC quantification could even be increased by a duplex PCR-assay, carried out subsequently after a negative enrichment (153).

A comparison of ISET technology for CTC detection and real-time PCR resulted in a statistically significant agreement of the two methods in their detection frequency (154), but a substantial variation in detection rates was seen comparing real-time PCR with the CellSearch® system and the AdnaTest, in which real-time PCR with CK19 and hMAM seemed to be the most sensitive of the three methods investigated (76).

In clinical routine it would be of great importance, to correlate gene expression values to a disease prognosis. It was found that expression of the three genes STC-1, GalNacT and MAGE-3 could be correlated to the sentinel lymph node metastasis (155), and the expression of the 'brain metastasis selected markers' (BMSM), Her2, EGFR, HPSE and Notch1 in EpCAM negative CTCs gives a hint towards brain metastasis (156). Furthermore, in a number of studies the correlation of CTC incidence and treatment efficiency was regarded using real-time PCR. The presence of CK19-mRNA-positive cells before front-line chemotherapy seemed thereby to be linked to a worse progression-free and overall survival (157), and the reduction of CK19-mRNA positive tumour cells by chemotherapeutic treatment was associated with a better survival in metastatic breast cancer patients (158). Different effects of taxane-based and taxane-free chemotherapy regimens were also seen in the presence of CK19-mRNA-positive cells and thus on patient prognosis. Taxane-based chemotherapy eliminated more CTCs than the taxane-free therapy, thereby contributing to a better disease-free survival (159). Other studies used additional marker genes for CTC-detection in therapy monitoring, for example Wang *et al* (160) used EpCAM, Her2, Ki-67, hTERT and vimentin in addition to CK19, and found indeed no correlation of the expression of those marker genes to TNM-stage of the tumour but was able to show, that the consideration of such markers could help in selecting appropriate therapeutics and for monitoring treatment efficiency. A combination of EpCAM, Her2 and Muc-1 also gave insights into the correlation of CTC incidence and therapy outcome. It was confirmed again, that the presence of CTCs after chemotherapy may classify a high-risk subgroup of patients, with a high probability of development of remote metastasis (161). These findings were again affirmed by Mikulova *et al* (162), who used TOP1, TOP2, CTSD, ST6 and CK19 as marker genes. Also heterogeneity in the expression of these genes in different patients was found, and it was concluded, that CTC detection is particularly useful for therapeutic decisions. Also marker genes for epithelial-mesenchymal transition, TWIST1, SNAIL1, SLUG, ZEB and FOXC2, were tested as real-time PCR marker genes, and it was shown that neoadjuvant therapy is unable to eliminate CTCs expressing these markers (163). For luminal subtype breast cancer patients, a combination of the marker genes MET and CD47 seemed to be helpful for risk stratification and treatment decisions (164).

Considering the research done in recent years it has become incresingly clear, that the process of epithelial-mesencymal transition has to be considered in CTC enrichment and marker gene selection. When an enrichment of CTCs is done by EpCAM it was recommended, to use a combination of different anti-EpCAM-antibodies, as it was shown, that even different breast cancer cell lines have different EpCAM expression (165). Therefore, it is crucial to regard also EMT-marker genes, as for example lymph node metastases show a strong expression of these genes (166), and a coexpression of EMT-marker genes VIM and Slug is found in patients with CK19-negative CTCs, underlining the importance of focussing on a wider spectrum of marker genes for CTC detection and characterization (167).

A future research topic in the breast cancer CTC-field will be the analysis not only of mRNA but also of miRNA expression profiles by real-time PCR, as well in the primary tumour as in CTCs (168) and also the investigation of plasma DNA (169). Another rather recent finding was, that CTCs might be retained in the microvasculature of the lung in a number of patients with metastatic breast cancer, leading to tumour cell emboli and dyspnoea (170).

Recent developments for CTC detection in ovarian cancer. One of the first approaches to detect CTCs from peripheral blood samples of ovarian cancer patients via real-time PCR was carried out in 2002, using CK7 and CK20 as marker genes. Although tumour cells were detected in some of the analysed blood samples, a conclusion could not be drawn between the incidence of CTCs and clinical and biological significance of these findings (171). A few years later Kallikrein genes six and ten were used for the detection of tumour cells in peripheral blood samples of ovarian cancer patients, but it was seen that the expression of these genes is not strong enough for CTC detection by real-time PCR (172). It is well known, that the presence of CTCs is linked to the occurrence of recurrences and disease-free and overall survival time (173,174) and generally poor clinical outcomes (175). Unlike in breast cancer, the correlation of CTC occurrence before and after chemotherapy was analysed also in ovarian cancer. It was again found, that the presence of CTCs, independent of the time-point of analysis, has no correlation to clinical parameters such as tumour size, grading and tumour histology (176), but is clearly linked to a shorter overall survival (177). Just like in breast cancer studies, also in ovarian cancer a further characterization of CTCs by use of more marker genes, like PPIC, is pursued (178). A rather new topic in the CTC-field in ovarian cancer is the analysis of invasive CTCs (iCTCs), as it was shown that iCTCs are better correlated to progression-free and overall survival and even more specific than the up to now widely used serum marker CA125 (179). The predictive value of CTC occurrence in ovarian cancer was reconfirmed in 2015 in a study of Zeng *et al* (180), who was able to demonstrate, that patients with CTCs in the peripheral blood had a shorter DFS and OAS than patients without CTCs in their blood.

Significance of CTCs in cancers of the cervix uteri. CTCs in patients with cervical cancer were already found in 1969, even in patients with 'carcinoma *in situ*' (181). This was confirmed in 2002, as the presence of CTCs in early stage cervical cancer samples was analysed by real-time PCR using CK19 as marker gene (182). It was concluded, that tumour cell dissemination is one of the early steps in tumour progression. Thereby CTC-detection might also in this cancer entity become an important tool for early estimation of tumour progression (183). The tumour cell retention in the microvasculature of the lung, which is reported for metastatic breast cancer was also shown to occur in cervical carcinomas (170).

CTCs and metastasis in endometrial adenocarcinoma. Up to now there are only a few studies dealing with CTCs in high risk endometrial adenocarcinoma. CTCs were isolated from blood of grade 3 patients and healthy control persons. CTCs were enriched by EpCAM based immunoisolation and analysed by real-time PCR. First, a high plasticity in expression of EMT marker genes ETV5, NOTCH1, SNAI1, TGFB1,

ZEB1 and ZEB2 could be shown, an association of CTCs with stemness was demonstrated by the expression of ALDH and CD44 and furthermore a potential for therapeutic targets was indicated by the expression of CTNNB1, STS, GDF15, RELA, RUNX1, BRAF and PIK3CA (184). Recently, the presence of CTCs in patients with high-risk endometrial cancer was also reconfirmed (185).

Further potential real-time PCR marker genes in breast cancer and different gynaecological malignancies. Mammaglobin was used as a real-time PCR marker, not only in breast cancer, but in a diversity of gynaecologic malignancies, like malignant tissue from the ovary, uterus and cervix. The expression of h-Mam could not only be detected from blood samples, but also from pleural or peritoneal effusions of cancer patients, while no expression could be detected in control samples from healthy volunteers, thereby making h-Mam a rather useful marker gene for real-time PCR based detection of circulating tumour cells (186). A more recent study aimed at finding a set of marker genes, which could be useful for CTC-detection from a variety of gynaecological malignancies. The six genes CCNE2, MAL2, EMP2, SLC6A8, DKFZp762E1312 and PPIC showed the highest detection rates in primary and recurrent breast, ovarian cancer, endometrial adenocarcinoma, and cervical cancer, and seem thereby to represent a sophisticated marker panel for CTC detection from blood samples of patients with gynaecological malignancies (187).

4. Conclusion

Real-time PCR is a highly sensitive method not only for detection, but also for characterization of minimal residual disease from blood samples of patients with epithelial cancer. To further verify the results of real-time PCR in respect to tumour cell quantification, it would be reasonable, to analyse blood samples simultaneously with a gold standard method, for example the CellSearch® system and real-time PCR. Additionally multiplex real-time PCR approaches could also increase the use of this sophisticated technique for tumour diagnostics and therapy monitoring. In a multiplex real-time PCR assay the gene expression of up to 4 genes can be examined simultaneously in only one PCR reaction, meaning, that with a rather small amount of patient material a reasonable detection and a simultaneous characterization of CTCs can be done. The characterization of tumour cells which is possible by real-time PCR approaches will clearly help to refine tumour diagnostics and treatment, as not only the primary tumour can be treated but also these residual tumour cells, which are the origin of distant metastasis. Thereby a future metastasis formation could be prevented or minimized. The CTC characterization could in term contribute to a reduction of side-effects of tumour therapy, increase the efficiency of treatment and lead to a more personalized tumour therapy.

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