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Detection and characterization of circulating and disseminated tumor cells in ovarian cancer to improve personalized therapeutic strategies

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1. Introduction

1.1 Ovarian cancer

1.1.1 Epidemiology and etiology

Ovarian cancer, a malignant proliferation of the ovary, is considered as the most lethal gynecologic malignancy worldwide [1]. In Germany 7,380 women suffered from this tumor entity in 2012 and about 5,646 died, indicating the highest case-to-fatality ratio in gynecologic malignancies [2,3]. According to the Robert Koch Institute (RKI), one in 72 women will develop ovarian cancer throughout her lifetime and the average age at onset of the disease is 69 years [2]. The relative five-year survival rate of patients with ovarian cancer is 41%, which is low compared to patients suffering from cancers in other reproductive organs e.g. cervical cancer with a relative five-year survival rate of 68% [2,4].

Risk factors fostering the development of ovarian cancer are either lifestyle-related or intrinsic, person-specific factors. Among the lifestyle-related risk factors obesity, childlessness (being nulliparous), or infertility and hormone replacement therapies increase the possibility of getting ovarian cancer [2]. In contrast, many full-term pregnancies, longer breastfeeding, hormonal oral contraceptives and sterilization by closure of the fallopian tubes (tubal ligation) reduce the probability to develop the disease [2]. Factors that cannot be influenced are age, polycystic ovaries, endometriosis, as well as family history. Regarding the last aspect, women whose first degree relatives had breast or ovarian cancer and women who themselves have already suffered from breast, corpus uteri or colon cancer have an increased risk for ovarian cancer [2].

About 90% of all ovarian cancers arise spontaneously and only 5-10% can be attributed to inherited genetic dispositions; here, mutations in the oncogenes BRCA1 and BRCA2 (breast cancer 1/2, early onset) are most frequent [2,5]. These mutations increase the risk of getting the disease significantly.

1.1.2 Histological types and classification

Ovarian tumors are known to originate from three different cell types: epithelial, germ, and sex cord stromal cells. With 90% of all ovarian tumors, epithelial cells are the most common [6]. Due to their histological characterization, they are subdivided into serous (most common), mucinous, endometrioid, clear cell, squamous, mixed, and undifferentiated subtypes [7,8]. In the past, it was thought that epithelial ovarian cancer has its origin solely on the surface of the ovary. However, new evidence proposes that the

fallopian tube and primary peritoneal cancers also resemble epithelial ovarian cancers [7]. In addition to the histopathological subtyping, ovarian cancer cells are assessed by comparing their morphology to that of benign ovarian cells. If the cancer tissue looks similar to healthy tissue (i.e. it consists of many different cell types that are grouped together) it is classified as a low-grade tumor. In contrast, less or un-differentiated tissue is denoted as a high-grade tumor. Furthermore, ovarian cancer is classified by the FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) classification using histopathological and pathological anatomical criteria to describe the position of the tumor and if it has spread to distant organs (FIGO stage I-IV) [9]. FIGO stage I and II are referred as early ovarian cancer, whereas FIGO stage III and IV are designated as advanced disease.

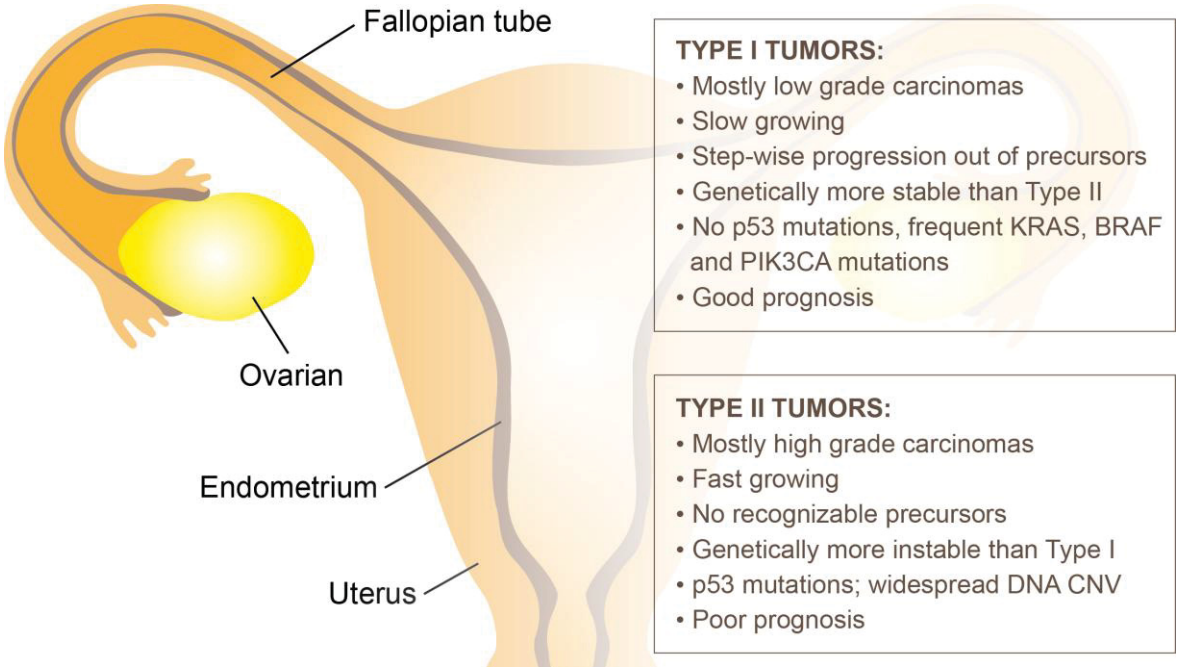

Figure 1: Types of ovarian cancer. Based on the histological type, differentiation status and molecular genetic aspects, ovarian cancers are grouped into two main categories (Type I and Type II) with different aggressiveness and prognosis. Type I tumors are low-grade, slow-growing carcinomas that develop from precursors lesions (borderline tumors). They are genetically stable and have a good prognosis. In contrast, Type II tumors are high-grade and fast-growing carcinomas, which are genetically unstable. At the time of diagnosis they have spread already beyond the ovary and dispose of a poor prognosis. CNV, copy number variation. Modified after [6] and [11].

Based on the histological type, differentiation status and molecular genetical aspects, ovarian cancers are grouped into two main categories (Type I and Type II) with different aggressiveness and prognosis [6,10,11]. Type I tumors grow slowly, are usually diagnosed at early stages and evolve stepwise from precursors, mostly borderline tumors that themselves have arisen from the ovarian surface epithelium, endometriosis or inclusion cysts [6,10]. Low-grade serous carcinoma, low-grade endometrioid carcinoma, mucinous carcinoma, and some clear cell carcinomas belong to this category [6]. Type I

ovarian tumors are genetically stable and do not show p53 mutations, while KRAS, BRAF and PIK3CA mutations are frequent [6]. In general Type I tumors dispose of a good prognosis [6,10,11]. In contrast, Type II tumors are fast-growing and belong to advanced (undifferentiated) stages. They contain high-grade serous and endometrioid carcinomas, undifferentiated carcinomas and some clear cell carcinomas. These tumors are genetically unstable and frequently harbor p53 mutations. Moreover, they show widespread DNA copy number alterations and are highly aggressive, resulting in a poor prognosis [6,10,11].

1.1.3 Therapy

Treatment of epithelial ovarian cancer (EOC) is mainly based on the combination of an initial debulking surgery and subsequent chemotherapy. Since the residual postoperative tumor load is one of the most important prognostic factors for the outcome of ovarian cancer surgeons strive for a macroscopic complete resection [12].

The five-year survival rate for early-stage (FIGO stage I or II) ovarian cancer is around 90% and in most cases surgery is sufficient to cure the malignancy [7,10,13]. However, in some cases the administration of *platinum*-based chemotherapy is recommended. The standard treatment of advanced epithelial ovarian cancer (EOC) combines surgical tumor debulking and a subsequent platinum-based chemotherapy, preferably consisting of carboplatin and paclitaxel [13,14]. Moreover an additional treatment with bevacizumab, an angiogenesis inhibitor, which reduces the growth of new blood vessels should be considered [10,13]. Recently, the PARP inhibitor Olaparib was approved for certain ovarian cancer subtypes. It inhibits the poly ADP ribose polymerase (PARP), an enzyme involved in DNA repair and is restricted to patients with hereditary BRCA1 or BRCA2 mutations [10].

Although over 80% of the patients benefit from first-line therapy more than half of them relapse shortly after an initial response to therapy (median 15 months) and will require a second-line treatment [7,10,15–17]. Patients whose cancer is progressing or are stable during first-line treatment or which relapse within 1 month are considered as '*platinum-refractory*', while patients who respond to initial treatment and relapse within 6 months are denoted as '*platinum-resistant*'. Besides, patients with a relapse more than 6 months after termination of first line therapy are characterized as '*platinum-sensitive*' [10].

Platinum resistant and refractory patients are therefore recommended for second-line treatment, which basically is a single-agent therapy typically with one of the following therapeutics: topotecan, gemcitabine or pegylated liposomal doxorubicin [13]. It should be

mentioned, that second-line treatments are just able to improve survival and quality of life, but cannot cure the disease [7].

1.1.4 Screening and monitoring tests for ovarian cancer

So far possible screening/monitoring tests for ovarian cancer are restricted to transvaginal ultrasound and a blood test for presence of the membrane glycoprotein *CA-125* (cancer antigen-125), which have been developed to detect the disease at an early stage or to monitor therapy effectiveness [8,13]. The former examination method can help to search for a conspicuous cell mass in the ovary but it does not allow a clear diagnosis [13]. Moreover, the identification of potential metastasis at distant sites is not possible.

Although the evaluation of the presence of CA-125 (also known as Muc-16) in the patients' blood is not recommended as an ovarian cancer screening method [13]. It is used to monitor the response to first- and second-line therapy, as an effective response to treatment is accompanied by a decreased CA-125-level and to detect residual or recurrent disease in patients who underwent first-line therapy [13].

However, high levels of CA-125 might have different reasons and only 80% of ovarian cancers express CA125. Thus, CA-125 alone is not considered as an ideal biomarker [8].

1.1.5 Route of dissemination/metastasis in ovarian cancer

In ovarian cancer, dissemination/metastasis occurs either by direct expansion of the primary tumor to adjacent organs (e.g. bladder and/or colon) or cancer cells detach from the primary tumor and are transported by physiological movement of the peritoneal fluid throughout the whole peritoneum within the abdominal cavity [3]. In contrast to most other organs, the ovaries are not confined by anatomical barriers, which in turn facilitates a widespread metastasis [3,8]. Additionally, detached tumor cells can transit through the lymphatic system to nodes at the renal hilus or through blood vessels to the parenchyma of the liver or lung [8].

1.1.6 Possible explanations for poor survival and future directions

Reasons for the poor survival rate of patients with ovarian cancer are manifold and can be linked to the lack of suitable detection/screening methods and/or specific symptoms. Consequently, most tumors are diagnosed at an advanced state (approx. 75%), which correlates with a decreased survival rate [2,3]. Besides that, more than half of the patients will relapse shortly after an initial response to chemotherapy [15–18]. Current therapy options do not consider important characteristics of the disease such as tumor heterogeneity (see 1.2) or the presence of chemo resistant tumor cells (termed cancer

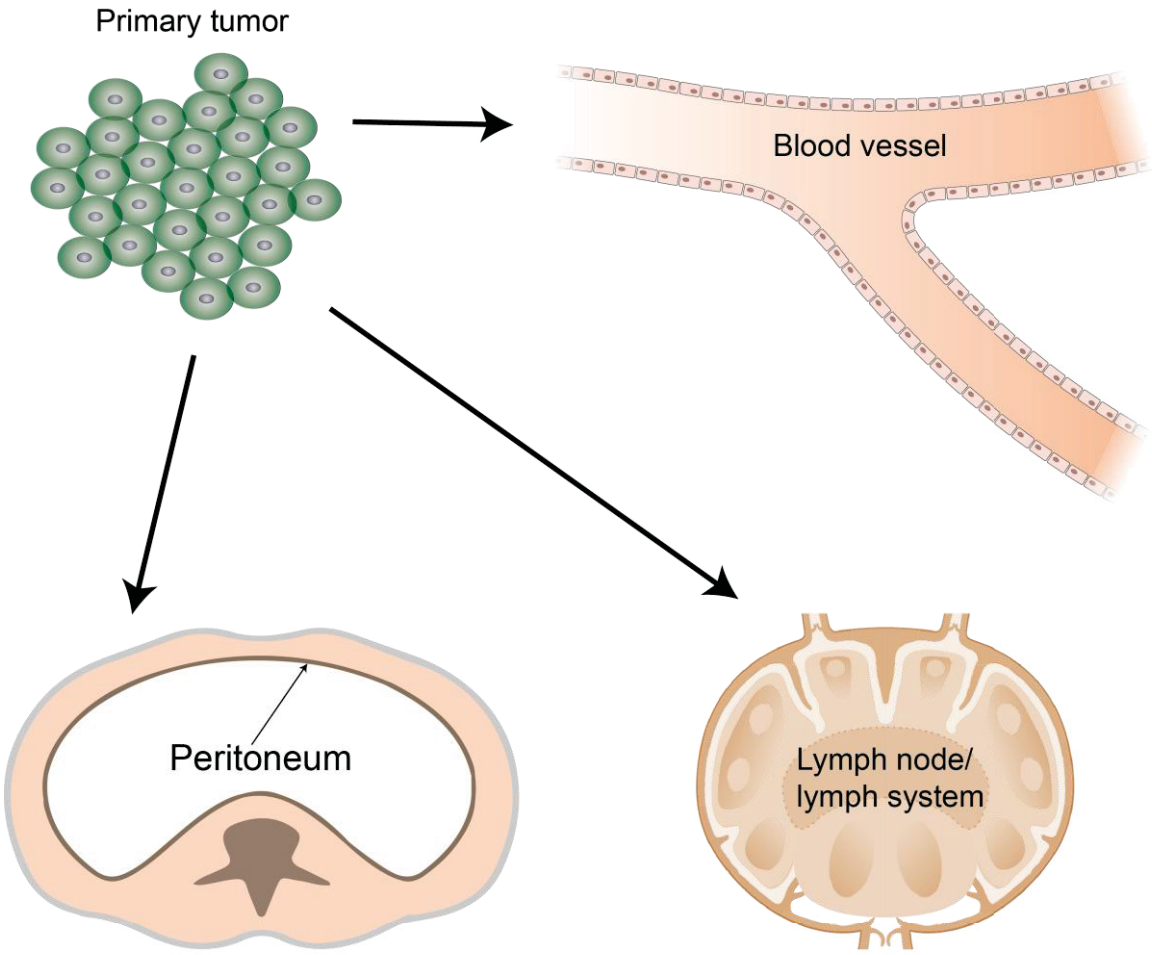

Figure 2: Route of dissemination in ovarian cancer. In ovarian cancer, tumor cells either detach from the primary tumor and get transported by the peritoneal fluid throughout the whole peritoneum within the abdominal cavity or transit through the lymphatic system to nodes at the renal hilus or through blood vessels to the parenchyma of the liver or lung.

stem cells (CSCs) (see 1.3)), which might explain treatment failure. Furthermore, there is increasing evidence that distant metastases display phenotypic and genotypic alterations compared to the primary tumor [19]. However, the biopsy of solid metastases for analysis is an invasive process, and most organs are not easily accessible. Though it was demonstrated that during tumor growth cancer cells detach from the primary tumor and circulate through the blood system (see 1.5) to extravasate at distant sites e.g. the bone marrow (see 1.6.), where they can survive chemotherapy and/or subsequently form metastasis. Therefore, the analysis of these cells is of high interest. The main advantage is, that blood and bone marrow are more easily accessible as organs and for the former the withdrawal is additionally non-invasive and can be performed repeatedly. Using these so-called liquid biopsy samples for the detection and characterization of residual cells may therefore not only advance the monitoring of ovarian cancer progression, but also the evaluation of therapies in individual patients. Eventually, these improvements will help to

develop alternative therapy strategies, tailored to the characteristics of disseminated cells that survived harsh medical treatment.

1.2 Heterogeneity of primary tumors

A major barrier to cure cancer, also ovarian cancer, is tumor heterogeneity. This heterogeneity can occur between primary tumors (inter-tumor heterogeneity) or within individual cells of the same primary tumor (intra-tumor heterogeneity). As mentioned above, ovarian cancer represents a diverse group of tumors, which are classified according to their histological subtype and response to therapy [1]. The latter is attributable to the heterogeneous mixture of functionally distinct tumor cells with unique properties. These functional varieties are the result of different receptor activities, as well as distinct metabolic, epigenetic and differentiation states, which can significantly affect treatment response [20]. For instance, mutations or overexpression of gene loci coding for the EGFR (epidermal growth factor receptor) or HER2 (Receptor tyrosine-protein kinase erbB-2) receptor can impair therapies targeting this receptor as they are used for non-small cell lung cancer (NSCLC) and colorectal cancer [20–22]. Another example for gene expression changes in this context is epigenetic modulation of the chromatin structure induced by DNA methylation or histone acetylation [20]. It is assumed that chemotherapy selectively promotes, either clonal expansion of intrinsically resistant and pre-existing resistant tumor cells or conversion of chemo sensitive tumor cells into a chemo resistant state and acquire ‘de novo’ therapy resistance under this selective chemotherapy pressure. Besides, it is argued that both mechanisms can co-exists [20,23,24].

1.3 Cancer stem cells

During the last two decades, evidence was found, that tumors might feature a small stem cell–like subpopulation. These so-called cancer stem cells (CSCs) are regarded as the source of metastatic tumor spread and have the capacity to enhance tumorigenesis and drug-resistance [25–29]. Their occurrence was documented in several cancer entities [27,30] and could be correlated e.g. with increased lymph node metastasis, advanced tumor stage and a shorter overall survival (OS) [27].

According to the American Association for Cancer Research (AACR) a CSC is defined as a cancer cell with a stem cell phenotype that owns the ability to self-renew, differentiate into different cell types, propagate tumors *in vivo* (generate xenografts) which reflects the heterogeneity of the primary tumor and is chemo- and radio resistant [25,27,31–33]. Due

to their capability to induce tumors *de novo* CSCs are alternatively named '*tumor-initiating cells*' [27]. Two major techniques are used to prove a CSC phenotype. The *in vitro* colony-forming assay is used to enrich CSCs and to verify their capacity for self-renewal. Here, the cells grow as multicellular spheroids under non-adherent conditions many days after initial seeding as a single cell suspension. Additionally, xenografting of isolated/enriched CSCs into a mouse model demonstrates the ability of tumor formation [25,27].

Similar to normal stem cells (NSCs) CSCs have the intrinsic ability to self-renew and differentiate and can be identified by stem cell markers such as CD133, CD44, and ALDH1 [27]. NSCs dispose of a strict control system of stemness pathways, which regulate important biological processes (e.g. embryogenesis and homeostasis). In contrast these pathways are deregulated in CSCs [27]. Another difference is that NSCs, especially those associated with the gastrointestinal tract and hematopoiesis, yield in terminally differentiated short-live cells and non-proliferative progenies, while descendants of CSCs dispose an unlimited proliferative and survival potential [27,34].

Besides their ability to self-renew CSCs are resistant to radiotherapy and chemotherapy [27,30]. Similar to NSC, CSCs are resistant to DNA damages and/or apoptosis [35,36]. High amount of free-radical scavengers reduce the concentration of reactive oxygen species (ROS) in CSCs, which is believed to render these cells even less prone to DNA damage [27,37]. Moreover CSCs possess the ability to overexpress ATP-binding cassette (ABC) transporter proteins which enables them to efflux chemotherapeutic drugs from their cytoplasm [25].

Current treatment strategies for ovarian cancer target the DNA, as they cross-link the DNA (*platinum*) or the proliferation capacity, by inhibiting the cell division (*taxanes)* of the cells [38]. Since NSCs are constitutive quiescent CSCs might also turn into a dormant state, enabling them to evade chemotherapy and leading to tumor recurrence, metastasis, and therapy resistance [25]. Moreover, it is assumed, that these tumorigenic and drug resistant cells are selected and enriched by current therapies, which in turn accelerates progression of the disease [25,31,39–41].

The identification and characterization of CSCs may offer novel targets for therapeutics, which can be combined with conventional therapies to completely eradicate cancer. Although several articles describe the isolation and detection of putative CSCs in different cancer entities, a defined marker set which reflects the profile of a typical 'stem-like' cancer cell has not been published yet [20,25,31]. As more than one population of CSCs within a tumor seems to be present and because it is not clarified, if CSCs arise from stem or progenitor cells, the identification of suitable markers is challenging [25]. Additionally,

there is accumulating evidence that tumor cells exhibiting stem cell features can develop from non-stem-like cells due to environmental stress or nutrient changes [42,43]. This is in accordance with the hypothesis that tumor cells with stem cell-like properties do not express a sharply defined phenotype, but rather represent a continuum with a high degree of plasticity. At least partly, such cell-state dynamics are thought to be responsible for the aggressive nature of tumors, and confer tumor-seeding capacity and drug resistance to these cells [42–44].

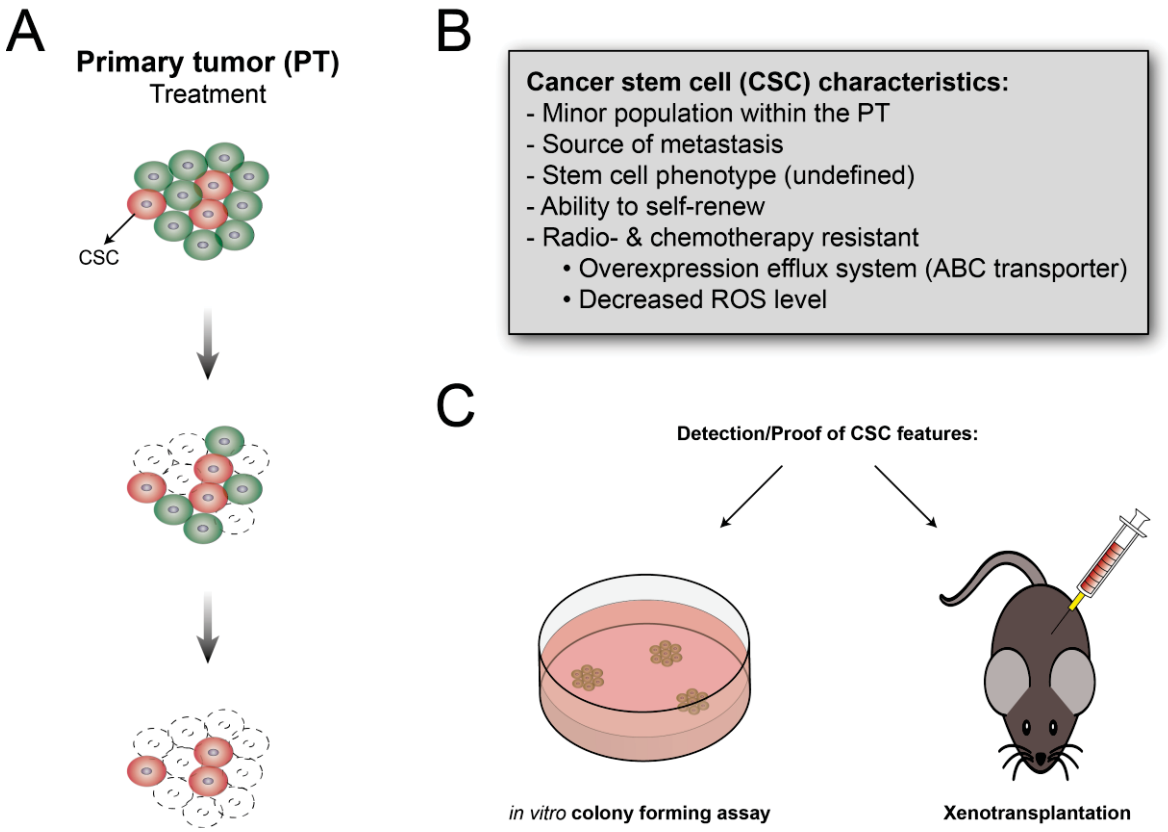

Figure 3: Cancer stem cells. Cancer stem cells (CSCs) are a small subunit of cancer cells with a stem cell-like phenotype, which are thought to be the source of metastasis. They are also believed to be chemo- and radio resistant as they overexpress efflux systems for chemotherapeutics and show decreased ROS levels (B). CSCs may be selected and enriched by current therapies, which potentially accelerates progression of the disease (A). Their ability to self-renew and to propagate tumors *in vivo* is proven by the *in vitro* colony forming assay and xenograft models (C). PT, primary tumor; ABC transporter, ATP-binding cassette transporter.

1.3.1 Cancer stem cells in ovarian cancer

Although the phenotype of ovarian CSCs has not been completely defined, various groups have already suggested protocols for the isolation and identification of CSCs from ovarian tumors and cell lines. Most frequently, the surface antigens CD133, CD44 and CD117 were selected as putative CSC isolation markers [1,31,45,46].

Typical CSCs features such as the ability to actively remove DNA binding dyes/chemotherapeutics from the cytosol, to self-renew and to propagate tumors *in vivo* could be confirmed [47–49]. Besides these typical CSC features proliferation assays and RT-PCRs of CSCs revealed an up-regulation of the stem cell markers B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), stem cell factor (SCF), Notch1, Nanog and octamer-binding-transcription factor 4 (Oct4) under stem cell-selective conditions [8,50]. Moreover, the progenitor nestin and the ATP-binding cassette G2 (ABCG2) were up-regulated in cells with enhanced chemo resistance to cisplatin or paclitaxel [8,50].

The expression of other pluripotency-associated stem cell factors, such as Aldehyde dehydrogenase 1 (ALDH1), SRY (sex determining region Y)-box 2 (SOX2) and LIN-28, could be confirmed in subpopulations of different ovarian cancer cell lines and tissues [51–53]. Supporting the hypothesis of a stem cell-like phenotype, Bareiss *et al.* demonstrated that SOX2 is expressed in serous ovarian carcinomas (SOC) and can induce CSC properties [53,54]. In addition, SOX2 was reported to enhance migration and invasion of ovarian cancer cells [54,55]. Importantly, SOX2 overexpression was shown to be a poor prognostic marker in ovarian cancer [56] and seems to be involved in taxane resistance [54,57,58].

1.4 Metastasis

Even though huge efforts have been undertaken in the last years to improve diagnosis, treatment and surgical techniques, 90 % of all cancer deaths are the result of metastasis [59].

Currently, two models describing the process of cancer progression/metastasis are proposed [60]. In the linear model, clonal selection of aggressive tumor cells within the primary tumor provokes a ‘stepwise’ progression, before some cells leave the primary tumor. Whereas in the parallel model, tumor cells disseminate from the primary tumor before it reaches its malignant phenotype [60].

1.4.1 The metastatic cascade

The process of metastasis is highly complex, and it is the most poorly understood stage of cancer pathogenesis. In general, it describes the release of cancer cells from the primary tumor and their dissemination to other (distant) organs where they subsequently adapt to foreign tissue microenvironments and grow out to metastases. [61]. Five essential steps are distinguished during the metastatic cascade: (A) Dissociation and local invasion, (B)

intravasation, (C) circulation (transport), (D) extravasation, (E) dormancy and/or metastasis [62].

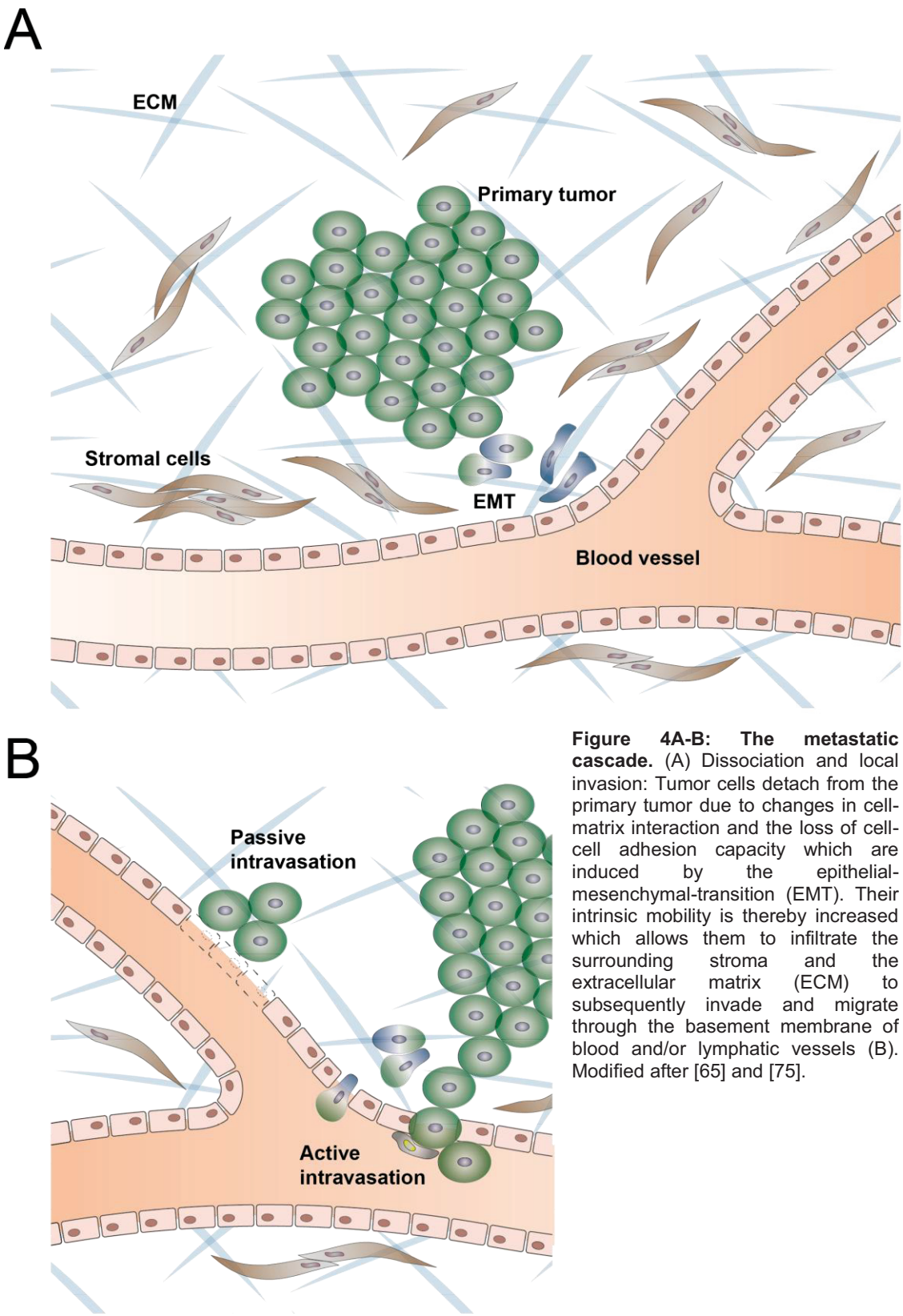

Figure 4A-B: The metastatic cascade. (A) Dissociation and local invasion: Tumor cells detach from the primary tumor due to changes in cell-matrix interaction and the loss of cell-cell adhesion capacity which are induced by the epithelial-mesenchymal-transition (EMT). Their intrinsic mobility is thereby increased which allows them to infiltrate the surrounding stroma and the extracellular matrix (ECM) to subsequently invade and migrate through the basement membrane of blood and/or lymphatic vessels (B). Modified after [65] and [75].

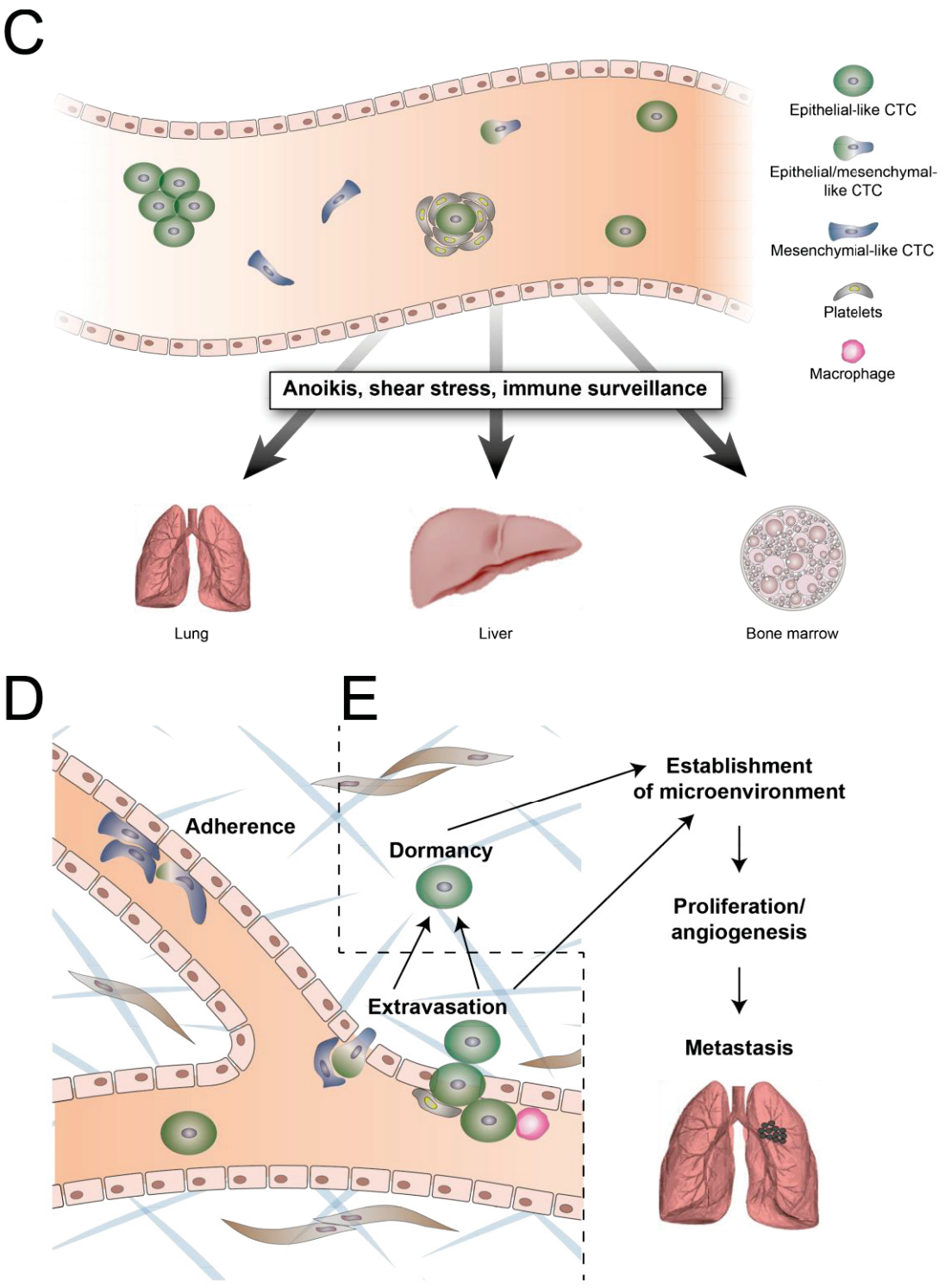

Figure 4C-E: The metastatic cascade. (C) Circulation/ Transport: The dissociated tumor cells enter the blood stream and circulate through the body. At this stage, they are designated as circulating tumor cells (CTCs). Subsequently, they are entrapped by small capillaries or attracted to distant organs where they leave the vasculature (extravasation) (D). Once cancer cells disseminated to distant organs, they either enter dormancy and/or proliferate to form metastasis (E). Modified after [65] and [75].

Dissociation

All solid epithelial tumors are embedded into a rigid extracellular matrix (ECM) and separated from benign tissue by the basal membrane. Both structures usually impede local invasion [63]. Like normal epithelial cells, epithelial tumors cells are characterized by a polarization of their plasma membrane proteins while they are interconnected by tight junctions, adherens junctions, gap junctions, desmosomes and integrins, consisting of adhesion molecules e.g. cadherins, claudins, or plakoglobin [63–65].

There are various assumptions described in the literature how cancer cells liberate from the primary tumor. Before they enter the blood stream they either actively invade the surrounding tissue or they are simply released [10]. In the latter case cell clusters separate from the primary tumor [66–69]. Tumor cells can then easily reach the lumen of the surrounding vasculature as tumor associated blood vessels are immature, leaky and poorly organized during angiogenesis [70,71]. In contrast, the major way for active invasion of tumor cells and subsequent intravasation into the bloodstream is based on the epithelial-to-mesenchymal transition (EMT) [72,73]. However, other forms of motility such as amoeboid movements additionally exist.

EMT

The process of EMT enables tumor cells to dissociate from the primary tumor due to changes in the cell-matrix interaction and loss of cell-cell adhesion capacity. Both processes increase the intrinsic mobility of the cells and allow them to invade the surrounding stroma [61].

EMT is a crucial step during a few fundamental biological processes, such as embryonic morphogenesis, wound healing and tissue regeneration. In order to form and/or repair tissues and organs, cells need to migrate within their local environment [65,74]. As outlined above, cancer cells can take advantage of EMT in order to leave the primary tumor.

The spectrum of EMT inducible factors is wide-ranging. For instance platelet-derived TGF-β as well as the direct contact of cells with platelets is known to trigger EMT. Interestingly, increased secretion of TGF- β was observed after ionizing radiation, which is also used in tumor therapies [43]. It has further been shown that interleukin-6 (IL-6), nicotine, alcohol, hypoxia and ultraviolet light affect the induction of EMT [65,73,75]. All these agents are thought to activate several transcription factors such as SNAI1/Snail 1, SNAI2/Snail 2 (also known as Slug), Twist, and Zeb which repress the transcription of the cell-cell adherent protein epithelial (E)-cadherin. E-cadherin is a key component of adherent junctions and enables cell-to-cell contacts by interconnecting their extracellular

domains. Down-regulation of E-cadherin leads to a loss of tight and adherent junctions, i.e. interconnections of adjacent cells and finally results in a loss of apico-basal polarity [72,75]. Simultaneously, mesenchymal markers such as vimentin and neuronal (N-) cadherin get up-regulated [71,76].

Although the process of EMT is mostly associated with primary tumor invasion, there is growing evidence, that it also influences the maintenance of CSCs. Is has been shown that differentiated epithelial cells might acquire stem cell-like properties when they undergo EMT [77,78]. This phenomena was confirmed by several research groups which observed co-expression of mesenchymal and stem cell markers in breast cancer cells [79–81]. Interestingly, EMT and the stem cell marker (ALDH1) expression was higher in patients who were resistant to therapy [80].

Local invasion

Besides the loss of the intercellular connection, remodeling of the surrounding stroma is necessary for local invasion [61]. During this process basal membrane/ECM degrading enzymes/proteases play an important role [61]. The protease urokinase-type plasminogen activator (uPA) proteolytically degrades ECM components and additionally activates multiple growth factors and matrix metalloproteinases (MMPs) that further contribute to ECM and basement membrane degradation. Hence, it is thought that these enzymes are key components for tumor cell invasion and intravasation [82,83]. Tumor-associated macrophages (TAMs) additionally promote tumor cell migration and intravasation by secreting chemotactic factors (e.g. epidermal growth factor (EGF)), remodeling the ECM, supporting angiogenesis and regulating the formation of collagen fibers [71,84] .

Intravasation/circulation/transport

In some tumor entities including ovarian cancer, metastasis occurs by direct expansion into body cavities [8,59]. However, in most cases, tumor cells usually intravasate into the bloodstream or the lymphatic system after local invasion [59,61].

Tumor cells that have entered the blood stream are designated as circulating tumor cells (CTCs, see 1.5). CTC clusters or single cells float freely or accompanied by blood cells (e.g. platelets) through the blood stream [61,85,86].

The persistence of CTCs in the blood is limited by several factors and their half-life is approximated to be in the range of 1–2.4 h [87]. It is assumed that most CTCs do not survive turbulence (e.g. circulatory shear forces, collisions with blood cells) and immune surveillance in the circulatory system. In addition, loss of contact with the primary tumor and substantial substrates of their microenvironment in the host tissue can induce anoikis (a form of programmed cell death) [59,65,68,88,89]. However, CTCs travelling in close

contact to platelets are protected against natural killer (NK) cell clearance [59]. Another known mechanism which helps CTCs to evade immune surveillance is the up-regulation of CD47, which shields CTCs from dendritic cell and macrophage actions [65,90].

Since both, mesenchymal and epithelial markers have been detected in the same CTC [91–93] it is supposed that this co-expression indicates an 'intermediate state' and that these cells do not completely lose their epithelial phenotype during EMT. More recently, it was shown that while primary tumor cells simultaneously express epithelial and mesenchymal markers, the mesenchymal cell phenotype is highly enriched in CTCs [43,91]. Additionally, a dynamic change in the relative numbers of CTCs harboring a mesenchymal or epithelial phenotype during targeted therapy was observed. Conspicuously, elevated CTC numbers as well as their mesenchymal phenotype could be correlated with treatment refractoriness and disease progression [43]. All these findings are in accordance with the hypothesis that CTCs do not express a sharply defined phenotype [44,91].

Extravasation/metastasis

Two fundamental pathways are proposed for the hematogenous and/or lymphatic extravasation of tumor cells, mechanical trapping of CTCs and the seed and soil hypothesis [94]. The former is believed to occur in capillaries with a low lumen to CTC diameter ratio. CTCs or CTC clusters entering narrow capillaries (e.g. arterioles), especially with a lumen smaller than their own diameter (approx. 4-30µm) are trapped and may cause a so-called tumor embolism [59,95–97]. Once they are trapped, single CTCs or clusters start to proliferate and form colonies, while others extravasate as solitary cells [59].

The seed and soil hypothesis postulates a direct/active interaction between the tumor cells (the 'seed') and the microenvironment of the preferred organ (the 'soil'), which contributes to a successful dissemination. For instance, breast cancer cells which express the cell surface protein metadherin specifically target the lung endothelium [59,98]. Furthermore, the expression of certain chemokines (e.g. SDF-1/CXCL12 and CCL21) in the lung, liver and bone marrow, which are all target sites of metastasizing breast tumors, is thought to attract CTCs [59,99].

In order to extravasate at distant sites, tumor cells have to permeabilize the endothelium of the blood or lymphatic vasculature. This so called transendothelial migration is usually observed for immune cells that leave the vascular system at a site of infection [61]. However, in particular aggregates (platelets + CTCs) can promote the adhesion of CTCs to leukocytes and endothelial cells of the capillary wall. Selectins, which are expressed on platelets, endothelial cells and leukocytes are crucial key adhesion molecules which allow

the docking (adhesion to endothelium) of the tumor cells to the endothelium by binding selectin ligands, which are expressed on the surface of the tumor [59,86,100]. This initial adhesion results in a release of cytokines and activation of integrins and the stable bound cells can move deliberately and finally actively migrate through endothelial cells [100]. Besides that, there is evidence that tumor cells secret soluble factors which induce retraction of endothelial cells, most likely by inducing apoptosis which in turn facilitates extravasation, [59,101,102].

During extravasation, tumor cells are supposed to undergo a mesenchymal-epithelial transition (MET) and regain their epithelial character [68,74,103]. Microenvironmental conditions determine the fate of disseminated tumor cells (DTCs), i.e. if they differentiate into a dormant state or directly form micrometastasis, which later on possibly develop into detectable macrometastasis [59].

The hypothesis that certain tumor entities preferably metastasize to specific organs has been postulated by Stephan Paget more than a century ago. It stated that the organ-preference patterns of tumor metastasis are the result of favorable interactions between metastatic tumor cells (the ‘seed’) and the microenvironment of the inhabited organ (the ‘soil’). His theory could be confirmed by the work of several research groups [59,104–106], which demonstrated that DTCs are seeded throughout the whole body, but they only develop metastasis at certain organs. This ‘organ tropism’ is highly variable and depends on the tumor entity. In general, lung, liver, bone and brain are frequent sites of metastasis formation [59,107]. In ovarian cancer, metastases preferably occur in the mesothelium of the peritoneal cavity, at the renal hilus or in the parenchyma of the liver or lung [8]. However, organ tropism has not been demonstrated yet for ovarian cancer, and specific organs are rather affected due to anatomical conditions [8,59].

1.5 Circulating tumor cells (CTCs)

1.5.1 Definition/characteristics

As already mentioned above, cancer cells are termed CTCs once they have entered the blood stream and originate from primary tumor tissue or metastatic lesions. Although millions of tumor cells are released from the primary tumor into the vascular system every day, most of them are eliminated by harsh environmental conditions, which is illustrated by their rather short half-life (1–2.4 h) [65,87]. As compared to white (few millions) and red (one billion) blood cells, their average abundance in one ml whole blood is quite low (about 1 - 10 CTC(s)) [108].

However, blood withdrawal can be used as a non-invasive liquid biopsy in order to detect and characterize CTCs and provide evidence about their frequency and phenotype. In addition blood examination might be a rapid and cost-effective alternative to organ biopsies and allows real-time monitoring of cancer therapies in individual patients and to screen for potential biomarkers [109].

According to the criteria defined by the CellSearch® system (see 1.5.2; the only FDA (Food and Drug Administration)-approved system for CTC enrichment and detection) a CTC is as a cancer cell with an intact, viable nucleus, and characteristics of an epithelial phenotype (constant expression of cytokeratins (CKs) and/or epithelial cell adhesion molecule (EpCAM). However, it has been shown that some CTCs co-express epithelial and mesenchymal markers [91–93]. Therefore, a more detailed definition for CTCs is needed. In most cases, CTCs feature a regular shape and have a diameter of 4 - 30 µm. [96,97,110]. However, another study included cells with an irregular shape or with subcellular morphologies [111]. Occasionally, single CTCs attach to each other and appear as CTC clusters, ranging from two to more than 50 cells (microemboli) [112] or CTCs are accompanied by leukocytes [68].

1.5.2 Enrichment of CTCs

As the ratio of CTCs to normal blood cells is very low, it is quite challenging to discriminate and detect true CTCs from benign cells in raw blood samples. Most methods therefore deploy a combination of enrichment and subsequent detection/identification (immunofluorescence (IF) and/or immunocytochemistry (ICC), target specific PCR) [113]. More than 40 CTC isolation/enrichment techniques are currently available [114], all of them exploit either specific physical (marker/label-independent approaches) or biological properties (marker/label-dependent approaches) [65,113,115].

Marker/label-independent CTC enrichment strategies isolate CTCs on the basis of physical features, such as density (Ficoll density gradient [116], OncoQuick™ [117]), size (different filtration technologies (e.g. ISET® [118,119], Parsortix [120])) and/or deformability/invasive capacity (Vita Assay™ [121,122]). The microfluidic device ‘JETTA’ captures CTCs by combining size- and deformability-based enrichment strategies. Moreover, it separates CTCs into single chambers which allows for subsequent single cell analysis [123]. Due to their specific electrical properties, size and shape, single intact CTCs can also be trapped into special electrical “cages” by applying a non-uniform electric field (a phenomenon also known as dielectrophoresis). Subsequently, CTCs can be recovered and manipulated (DEPArray™ System) [124].

Marker/label-dependent approaches are mainly directed against EpCAM (e.g. CellSearch® [125], CTC- [126] /HB (herringbone)- [91,127]Chip, IsoFlux® [128]) or a combination of this protein with other surface markers such as Muc-1 (cell surface associated Muc-1) (AdnaTest® [79,80,129–131]). All of them use ferrofluid/bead-coupled EpCAM antibodies to enrich CTCs. However, the CellSearch® approach is considered as the 'gold standard' for CTC enrichment and detection [65], since it was shown that CellSearch® enriched CTC counts negatively correlate with progression-free survival (PFS) and overall survival (OS) in primary [132] as well as in metastatic diseases [133]. Therefore, this system is most commonly used in clinical trials or clinical routine for breast, colon and prostate cancer [65]. However, it has not been approved for ovarian cancer [108]. All these techniques can only process a small amount of blood, which in turn limits the capability to detect low-concentrated CTCs, especially at an early stage of the disease [113]. Examples for methods which allow the analysis of larger blood volumes are the GILUPI CellCollector™ [134] or leukapheresis [113,135]. The former consists of an EpCAM-coated wire, which is placed into the vein of the patient and is capable of processing up to 1.5l blood within 30 minutes [65,113,134].

Limitations and alternatives

Not all cancer types e.g. ovarian cancer feature EpCAM-expressing CTCs, or EpCAM is expressed at a very low level. Moreover, tumor cells which undergo EMT down-regulate the expression of EpCAM and keratins, while the expression of mesenchymal markers is up-regulated [65,76,79,136]. Thus, alternative or additional (surface) markers are necessary to capture/enrich CTCs in these cancer entities. For instance, the AdnaTest (OvarianCancer) combines EpCAM- and Muc-1-dependent magnetic enrichment [15,129,131]. This combination was additionally tested in a microfluidic device which resulted in an improved capture efficiency [137]. Moreover, it is challenging to identify mesenchymal-like CTCs within the hematopoietic cell background of the blood, which is also of mesenchymal origin [65]. To facilitate the enrichment of CTCs one approach is, to deplete CD45 positive leukocytes [65]. For instance, the CTC-iChip combines CD45 depletion and subsequent size-based CTC enrichment [138].

Table 1: Enrichment and detection methods for circulating tumor cells

Technology	CTC isolation/enrichment	CTC detection/characterization	Reference(s)
Marker/label-independent			
Ficoll density gradient	Density gradient centrifugation	ICC, PCR, EPISPOT, FISH	[116]
OncoQuick™	Density gradient centrifugation	ICC, PCR	[117]
ISET®	Filter with pores >8 µm	ICC, PCR, FISH	[118,119]
Parsortix	Filter with pores 5-10 µm	ICC, PCR, FISH	[120]
VitaAssay™	Attachment and digestion of CAM matrix	ICC, PCR	[121,122]
JETTA™	Microfluidic chip, size- and deformability-based	ICC, PCR, FISH	[123]
DEPArray™	Dielectrophoresis (electric properties)	ICC, PCR, FISH	[124]
EPISPOT	Density gradient centrifugation	Immunological detection of secreted proteins (e.g. Muc-1, CK19) on an antibody coated membrane	[113,139]
Marker/label-dependent			
CellSearch®[1]	Immunomagnetic enrichment with EpCAM-ferrofluid	ICC (DAPI, CK & CD45), PCR, FISH	[125,132,133]
CTC-Chip	EpCAM coated microposts	ICC (CK/EpCAM, CD45), (RT)-PCR, FISH	[126]
Herringbone-Chip	EpCAM (HER2, EGFR) coated microposts	ICC (EpCAM, CK 5,7,8,18,19, CDH1/2, FN1, PAI1, CD45), PCR	[91,127]
IsoFlux®	Micofluidic device with magnetic isolation zones for EpCAM	(RT)-PCR, FISH	[128]
AdnaTest® OvarianCancer [2]	Immunomagnetic enrichment with EpCAM & Muc-1 beads	Multiplex-RT-PCR (CA125, EpCAM, Muc-1 & ERCC1)	[129–131]
AdnaTest® EMT-1/StemCell	Immunomagnetic enrichment with EpCAM & Muc-1 beads	Multiplex-RT-PCR (PI3K,Akt2,TWIST), Singleplex-PCR (ALDH1)	[79,80]
GILUPI CellCollector™	EpCAM-coated wire for *in-vivo* application	ICC	[134]
Marker/label-independent and marker/label-dependent			
Liquid bead array	Density gradient centrifugation and immunomagnetic enrichment with EpCAM coated beads (CELLection™)	Multiplex-RT-PCR (CK19, HER2, MGB1, MAGEA3,TWIST1 & HMBS)	[140]
CTC-iChip	Size and EpCAM-based selection / negative depletion of CD45 + leukocytes	ICC, PCR, FISH	[138]

ALDH, Aldehyde dehydrogenase; CA125, Cancer antigen-125; CAM, Cell adhesion molecule; CD45, Cluster of differentiation 45; CDH1/2, Cadherin-1/2 (E-/N-cadherin); CK, Cytokeratin; DAPI, 4',6-diamidino-2-phenylindole; EpCAM, Epithelial Cell Adhesion Molecule; EPISPOT, EPithelial ImmunoSPOT; ERCC1, Excision repair cross-complementation group 1; FISH, Fluorescence *in situ* hybridization; FN1= Fibronectin 1; HBMS, Heparanase; HER2, Human epidermal growth factor receptor 2; ICC, Immunocytochemistry; MAGE-A3= Melanoma-associated antigen 3; MGB1, Mammaglobin 1; Muc-1= Mucin-1; PAI1= Plasminogen activator inhibitor-1; PBGD= Porphobilinogen deaminase; (RT)-PCR, (Reverse transcription)-polymerase chain reaction; TWIST, Twist-related protein 1. The table is modified after [65].

[1] FDA approved for breast, colon and prostate cancer
[2] Available for breast, colon and prostate cancer

1.5.3 CTC detection/identification

The most common techniques used for the identification and enumeration of CTCs after enrichment are IF and ICC. Both methods are antibody-based approaches which preferable identify CTCs by the detection of epithelial markers such as cytokeratins (CK 8/18/19) and EpCAM [97,141]. Furthermore, DAPI (4,6-diamino-2-phenylindole) is used to visualize the nucleus. Application of the leukocyte antigen CD45 (cluster of differentiation 45) detection allows to discriminate CTCs from potentially co-enriched blood cells and should not be expressed on CTCs [96,113]. In order to identify only viable CTCs, the so called EPISPOT (EPithelial ImmunoSPOT) assay deploys an antibody-coated membrane that captures secreted proteins released from short time cultivated tumor cells. Captured proteins are then visualized by incubation with secondary antibodies [113,139]. As a high amount of markers of interest might complicate the staining with fluorescent dyes, the identification of CTCs could also be performed by multiplex-RT-PCR assays for several tumor-surface-associated and EMT-associated transcripts [65,79,142].

1.5.4 Characterization of CTCs

Besides the detection/identification and enumeration, the molecular characterization of CTCs is of high interest, since for example alteration of receptor expression can lead to aberrant oncogenic signaling and gene expression [112]. Detection of changes by molecular profiling of CTCs for genetic variants, gene and protein expression patterns has the great potential not only to identify new, therapeutically more relevant targets, but also to detect emerging resistances [112,143]. Therefore, the characterization of CTCs may enable to develop personalized treatment strategies and may be used for real-time monitoring of therapeutic efficacy [143,144].

Currently, there are several technologies tested to characterize CTCs [68]. They can be separated into DNA-, RNA-, and protein-based as well as functional approaches.

Genome

The oncogenic over-expression of genes which are of particular interest for targeted therapy strategies is often caused by the amplification or rearrangement of their genetic regions [112]. In order to identify a panel of different chromosomal regions or tumor-specific chromosomal rearrangements of target genes in CTCs fluorescence *in situ* hybridization (FISH) has been used [145]. Among others, genetic amplifications of HER2 in CTCs could be observed [146–148]. Several groups have already examined somatic mutations or other alterations in therapeutically relevant genes by using pooled or single CTCs from patients with various tumor types [149–152]. Mostly, regions of interest were amplified by gene specific PCR to enable downstream analysis. Preferably,

mutations/single nucleotide polymorphisms (SNPs) were analyzed with the help of SNaP shot analysis or HPLC (High-Performance Liquid Chromatography).

Due to the fact that only two copies of a chromosome are available in a single diploid cell [65], whole genome amplification (WGA) technologies were developed to increase the amount of DNA for subsequent single cell analysis. Using amplified genomic DNA, copy number alterations were determined by array comparative genomic hybridization (aCGH) [19,153–156], while (point) mutations were detected by next-generation sequencing approaches [68,153] (e.g. whole exome sequencing [157]). The disadvantage of these amplification steps is that they are susceptible to technical errors such as low physical coverage, non-uniform coverage and allelic dropout events, which may lead to false-positive and false-negative results [158,159]. DNA modifications such as promoter methylation(s), which can lead to epigenetic silencing of tumor suppressors (e.g. BRMS1, CST6 and SOX17) and directly influence biological properties and processes of tumor cells (e.g. proliferation, invasiveness, epithelial phenotype and stemness), can be examined by methylation specific PCR (MSP) [143,160].

Transcriptome

Studying gene expression patterns in CTCs is quite challenging and complex. For instance, RNA degradation, i.e. the loss of transcripts, is a major issue which has to be avoided. Therefore, already pre-analytical issues such as cell stability and viability during sample shipment as well as immediate processing of blood samples and non-harmful CTC isolation are crucial for subsequent gene expression analysis. [143,161,162]. To gain a clear and accurate picture about gene expression, the reverse transcription (RT)-(q)PCR has to be of high sensitivity and specificity, e.g. miss-priming and amplifications of pseudo genes have to be avoided [143].

Most groups investigated gene expression patterns of pooled CTCs with the help of standard RT-PCR [163,164] or real time PCR (RT-qPCR) [165–168]. In these studies, the analysis was restricted to only a small number of genes which were previously correlated with a possible therapeutic intension/information. Since researchers are interested in the analysis of various genes but CTCs are rare, they focused on the amplification (and quantification) of multiple gene-targets at the same time (multiplex-RT-(q)PCR) [142,143,165]. Data acquisition in studies presenting single or multiple gene expression analysis of CTCs is either based on custom-made protocols [165,169–171] or the commercially available product AdnaTest [15,130,131,142,172].

An alternative approach for gene expression analysis on CTCs is the liquid bead array technique [140]. The unique feature of this system is the biotinylation of all transcripts by PCR. All biotinylated amplicons are then hybridized to gene-specific capture probes,

which are immobilized on the surface of fluorescent microspheres. After incubation with streptavidin-phycoerythrin, amplicons are quantified by the Luminex flow cytometry system [140,143]. In the beginning, this method allowed a simultaneous detection of six gene-transcripts isolated from CTCs [140]. Nowadays it is capable of analyzing up to 100 genes and their splice variants [140,143,173].

Besides, an bead-array-based method was deployed to perform gene expression profiling of more than 29.000 genes on spiked breast cancer cells ([174].

Although the techniques listed above are established tools for the characterization of gene expression profile in CTCs, some limiting factors become more and more evident. For instance, CTCs are highly heterogeneous, even within the same patient [175]. Gene expression data from bulk samples might cause misleading interpretation, since the detected transcripts cannot be assigned to a specific CTC. In this context, detection of co-expressed transcripts within one cell are also not possible [143]. Additionally, even the most sophisticated isolation techniques are still not able to deplete all leukocytes, which may contribute to misleading observations and interpretations: due to their hematopoietic origin, leukocytes express EMT markers, additionally stem cell markers are expressed in incompletely differentiated leukocytes [176]. In contrast, single cell analysis of CTCs allows the discrimination of cells with different expression pattern.

Powell *et al.* developed a chip based high throughput qRT-PCR array for single cell profiling. In their work, they documented a high heterogeneity of breast cancer CTCs, even when the cells were isolated from the same patient. Moreover, they discovered a distinct profile heterogeneity between CTCs and cancer cell lines. This finding raises questions about the clinical relevance of screening assays which aim to identify novel therapy targets in cell lines [143,175]. Performing single cell RNA sequencing Ting et al, observed a similar phenomenon when they compared genome-wide expression profiles of single pancreatic CTCs and xenografted primary tumors. They noticed that CTCs clustered differently in single CTCs, primary tumors and tumor-derived cell lines [177].

Besides the aforementioned techniques, RNA-ISH (RNA- *in situ* hybridization) is an inexpensive and versatile tool for the detection and characterization of CTCs on the single cell level. For instance, the expression of various epithelial and mesenchymal markers was investigated by several groups [91,178,179]. In particular, a correlation between mesenchymal CTCs, disease progression and CTC cluster formation was observed [91]. As compared to the analysis on the genomic level, an advantage of RNA analysis in single cells is that thousands of copies of each mRNA transcript are available [180]. Although highly sensitive, amplification steps are still required in most RNA-based techniques, especially for array- and sequencing-based techniques [174,177].

Detection of EMT and stem cell like transcripts on CTCs

CTCs with stem cell characteristics could be confirmed on the transcriptional level in several tumor entities. For instance, CTCs with cancer stem cell characteristics (e.g. expression of CD133, ABCG2) were detected in patients with hepatocellular carcinoma [181]. Furthermore, the existence of breast cancer CTCs (co)-expressing EMT and/or the stem cell markers could be proven by the commercially available AdnaTest (EMT-1/StemCell) [79,81,143] or other RNA-based techniques [55,182–185]. These results strongly indicate that commonly used isolation/enrichment strategies which are based on epithelial characteristics exclude CTCs with a mesenchymal phenotype or an 'intermediate state' [79,80]. The great importance of these kinds of CTCs has additionally been demonstrated in a study published by Ueo *et al.*. The authors found that the EMT-related biomarker plastin3 (PLS3) is expressed in breast cancer CTCs and patients with PLS3-positive CTCs had significantly shorter OS and DFS [143,186].

Gene expression profiling of ovarian cancer CTCs

Our knowledge about the expression profile of ovarian cancer CTCs is rudimentary. Just a few publications focusing on this topic are available. All these studies pooled CTCs and potential contaminations with leukocytes might have interfered with signals from true CTCs. [15,187–190].

Additionally, in studies using the commercially available AdnaTest for the analysis of ovarian cancer CTCs are restricted to the detection of the epithelial markers EpCAM, Muc-1, CA-125 and the growth factor receptor HER-2 [15]. Kuhlmann et al expanded the test by the detection of the excision repair cross-complementation group 1 (ERCC1) gene transcript, which is used as a biomarker for the prediction of *platinum* resistance in ovarian cancer patients at the stage of primary diagnosis [143,190].

Another study identified eleven genes as markers for CTCs in EOC patients by comparing matched EOC tissues and peripheral blood leucocytes. Gene expression of these novel markers and EpCAM was further analyzed by RT-(q)PCR and it turned out that just one marker, namely cyclophilin C (PPIC) was overexpressed. Interestingly, PPIC positive CTCs were significantly more often detected in *platinum*-resistant than in *platinum*-sensitive patients [143,189].

Two of the above mentioned publications investigated gene expression by RT-qPCR using short-time cultured CTCs providing that the most frequently elevated genes in ovarian cancer CTCs (serous type) are EpCAM, CK19 and Muc-1 [187,188].

Proteom

The commonly used antibody-based approaches for CTC detection (ICC and IF) are also applied for the characterization of CTCs. Besides the detection of mutations in therapy-relevant targets (e.g. ER (estrogen receptor) and HER2 in breast cancer) even the expression of this genes was employed on CTCs by IF [191,192], not least to compare, if the expression/occurrence is similar to the primary tumor [193]. A very smart solution is the combination of protein detection by IF and gene amplification by FISH named FICTION [68]. One example is the simultaneous amplification of EGFR and determination of HPSE (heparanase) expression [194].

As already shown on the RNA level, the expression of epithelial and mesenchymal markers on CTCs was further confirmed on the protein level by IF [92,93,195]. By using IF and flow cytometry, stemness/CSC markers such as ALDH1 and CD44 were also found to be expressed on CTCs [182,183,196].

Functional assays

Functional *in vitro* or *in vivo* assays have been developed to mimic the evolution of metastasis and to screen for putative drugs. These models will help to gain further insights into the biology of CTCs, which in turn is believed to finally uncover metastasis-initiating cells. Viable CTCs can be detected either by the VitaAssay™ [120,121] (which additionally allows for the identification of invasive CTCs) or by the EPISPOT assay (which analyzes secreted proteins (e.g. cytokeratins) [113,139]. As only a small amount of CTCs can be isolated from the patients' blood, one big goal is to develop novel *in vitro* CTC culture technologies that foster the cell expansion. Some groups observed the formation of non-adherent tumor spheres by proliferating CTCs under specific culture conditions, such as serum-free media supplemented with certain growth factors (e.g. EGF) or hypoxia [194,197]. Moreover, CTCs isolated from a castration-resistant prostate cancer patient developed into three-dimensional organoids using a special *in vitro* cultivation technique [68,198].

A pioneering technological breakthrough regarding the expansion of CTCs was accomplished by Cayrefourcq *et al.* They were able to establish the first *ex vivo* colon CTC line, which showed high stability, resembled the cell characteristics of the primary tumor and induced xenograft tumors in immune-deficient mice [68,199]. Interestingly, this cell line has an intermediate epithelial–mesenchymal phenotype, with stem-cell like features and an osteomimetic signature, which implies a possible bone marrow origin [68,199].

Table 2: Characterization of circulating tumor cells. Single or pooled CTC(s) can be analyzed by genome-, transcriptome- and proteom-based technologies. Moreover functional analyses can be performed to validate their metastatic capability and to determine drug efficacy.

Characteristics	Technology	Reference(s)
Genome analysis		
Chromosomal rearrangements/gene amplifications	FISH	[145–148]
Somatic/genetic mutations, SNPs	Sanger sequencing SNaP shot analysis HPLC	[149–152]
Copy numer variants (CNV)	aCGH	[153–155]
Whole genome/exome mutation analysis	NGS (whole genome/exome sequencing)	[153,157]
Promoter methylation	Methylation specific PCR	[143,160]
Trancriptome analysis		
Single gene transcripts	RT-(q)-PCR Fluorescene RNA-ISH	[165,166,168,169]
Several/multiple transcripts	Multiplex-RT-(q)-PCR Liquid bead array technique Expression arrays RNA-sequencing Fluorescene RNA-ISH	[142,143,165,167] [140,173] [174] [177] [91,178,179]
Proteome analysis		
Protein expression	ICC/IF Flow cytometry	[92,93,191–194] [182,183,196]
Protein expression and gene amplification	FICTION (IF + FISH)	[68,194]
Secreted proteins	EPISPOT	[113,139]
Functional assays		
Viable CTCS	EPISOT, Vita Assay™	[113,139] [121,122]
Increase/enhance the amount CTS for potential drug testing	CTC cultivation as non-adherent tumor spheres Establishment of a cell line	[194,197] [68,199]
3D-models to proove the metastatic capacity and drug testing	3D- organoid models xenographts	[68,198] [136,200,201]

aCGH, Array comparative genomic hybridization; EPISPOT, EPithelial ImmunoSPOT; FICTION, Fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm; FISH, Fluorescence *in-situ* hybridization; HPLC, High-performance liquid chromatography; ICC, Immunocytochemistry; IF, Immunofluorescence; NGS, Next-generation sequencing; RNA-ISH, RNA in situ hybridization; RT-(q)-PCR, Reverse transcription-(quantitative) polymerase chain reaction; SNP, Single nucleotide polymorphism; WGA, Whole-genome amplification; WTA, Whole-transcriptome amplification. The table is modified after [68].

In vivo xenotransplantations of CTCs into immune-deficient/compromised mice are used to evaluate phenotypic changes of the cancer cells (e.g. the process of EMT). For example, Gorges *et al.* used a murine breast cancer xenograft model to analyze the EMT-status; the authors not only confirmed the loss of the epithelial marker EpCAM, but also observed an upregulation of mesenchymal markers on CTCs [136]. In another study *in vivo* tracking of fluorescently labeled tumor cells in an orthotopic xenograft model

revealed alterations in cell morphology and gene expression towards an EMT-like phenotype. Interestingly, this effect was more pronounced in recovered tumor cells, which were used for a second application of xenograft tumor formation [43,200]. Baccelli *et al.* transplanted breast cancer CTCs into immune-deficient mice to search for metastasis-initiating cells (MICs) and to examine their phenotype. They decoded a special expression pattern (EpCAM, CD44, CD47 and MET) within the MIC-containing CTC populations [43,201].

1.5.5 Relevance of CTCs in ovarian cancer

Several studies confirmed the presence of CTCs in ovarian cancer, and researchers have already started to enumerate and characterize these cells [202,203]. However, the question if CTCs actually harbor a prognostic potential in ovarian cancer is still subject to discussion. Many studies demonstrated that the presence of CTCs is associated with poor clinical outcome, i.e. OS and/or PFS/DFS (progression-free survival/disease-free survival) were significantly reduced [15,190,204,205], whereas other publications did not observe a correlation between CTC status and prognosis [206–208]. Moreover the detection/enumeration rates are very different and range from 12% to 83% [203]. Possible explanations are varying methodological procedures, non-uniform CTC definitions and different cut off levels for detection and quantification of CTCs across the studies [202,203]. Another reason might be that the presence of CTCs was determined at different time points [203,209]. Interestingly, the presence of CTCs after completion of adjuvant treatment was shown to correlate with shorter OS, indicating their negative prognostic impact [15].

1.5.6 Metastasis initiating potential of CTCs

Even though aggressive tumors release thousands of cancer cells every day, only a few metastases are detected in most patients. This clinical finding supports the theory that metastasis is a highly inefficient process [6]. This could be confirmed by Baccelli *et al.*, since more than 1,000 isolated breast cancer CTCs were needed for xenotransplantation into immunocompromised mice to induce metastatic outgrowth [112,201]. Nevertheless, a correlation between CTC counts and cancer progression and/or decreased overall survival was proven for several cancer entities [125,133,210–214], underlining the theory that a sub-population of metastasis-initiating cells are present among CTCs.

1.6 Disseminated tumor cells (DTCs)

Disseminated tumor cells (DTCs) are tumor/cancer cells that have left the primary lesion and reached a secondary organ. In general, their presence is defined as minimal residual disease, as they are not detectable by conventional routine diagnostic procedures. As homing site DTCs prefer the bone marrow (BM), where they can either form metastases (mainly in breast or prostate cancer) or initially persist/survive to subsequently re-circulate and move to other distant organs providing better growth conditions e.g. the lung or liver [215,216]. DTC persistence is supported by the fact that tumor cells are detectable within the peripheral blood even months or years after removal of the primary tumor [87,216,217]. It is known that tumor cells escape from the primary tumor in an early stage of the disease and that DTCs are detectable within the BM already at the time of diagnosis [209]. Moreover, DTCs might circulate back to the primary tumor site (before resection), which is also denoted as 'tumor self-seeding' and could be confirmed in mouse xenograft models for breast, colon and melanoma tumors [215,218,219].

1.6.1 Detection and characterization methods for DTCs

Similar to CTCs, DTC detection initially requires an enrichment step, which is commonly carried out by density gradient centrifugation and subsequent red blood cell lysis. Rather infrequent affinity-based separation approaches, e.g. removal of CD45 expressing cells by immunomagnetic depletion is used [216,220]. After DTC enrichment and negative depletion of any unwanted cells, samples (0.5 to $1.5x10^6$ mononucleated cells) can be centrifuged onto glass slides and analyzed for epithelial/tumor-associated markers by ICC [216,220]. Herein, pan-cytokeratins serve as standard epithelial markers to distinguish DTCs from cells of hematopoietic origin [76,215]. The antibody clone A45-B/B3 represents the ‘gold standard’ as it detects CK 8, 18, and 19 simultaneously [215,220,221]. To ensure, that cytokeratin-positive cells are indeed tumor cells, aCGH is the method of choice to detect genetic alterations [215,222,223]. In general, techniques used for CTC characterization can also be employed for DTCs (see 1.5.4).

Challenges in DTC detection and characterization

Unlike other organs, the BM is easily accessible to perform biopsies. Nevertheless, even though BM aspiration is routinely performed in clinical diagnostics, the withdrawal is an invasive process performed under anesthesia, making it less comfortable and acceptable to the patient than normal blood drawl. Besides, it is not feasible during control visits at outpatient centers and cannot be iterated several times [216]. To prevent the BM from mixing with blood, which could lead to false positive results and to gain higher yields, BM aspiration should be performed from the iliac crest [216]. Due to the hematopoietic origin

of the BM cells, the detection of DTCs with a mesenchymal phenotype is challenging. For the evaluation of stem cell markers on DTCs, labeling of the hematopoietic stem cell marker CD34 (cluster of differentiation 34), expressed by hematopoietic stem cells (HSCs), should be included to avoid false positive results [54].

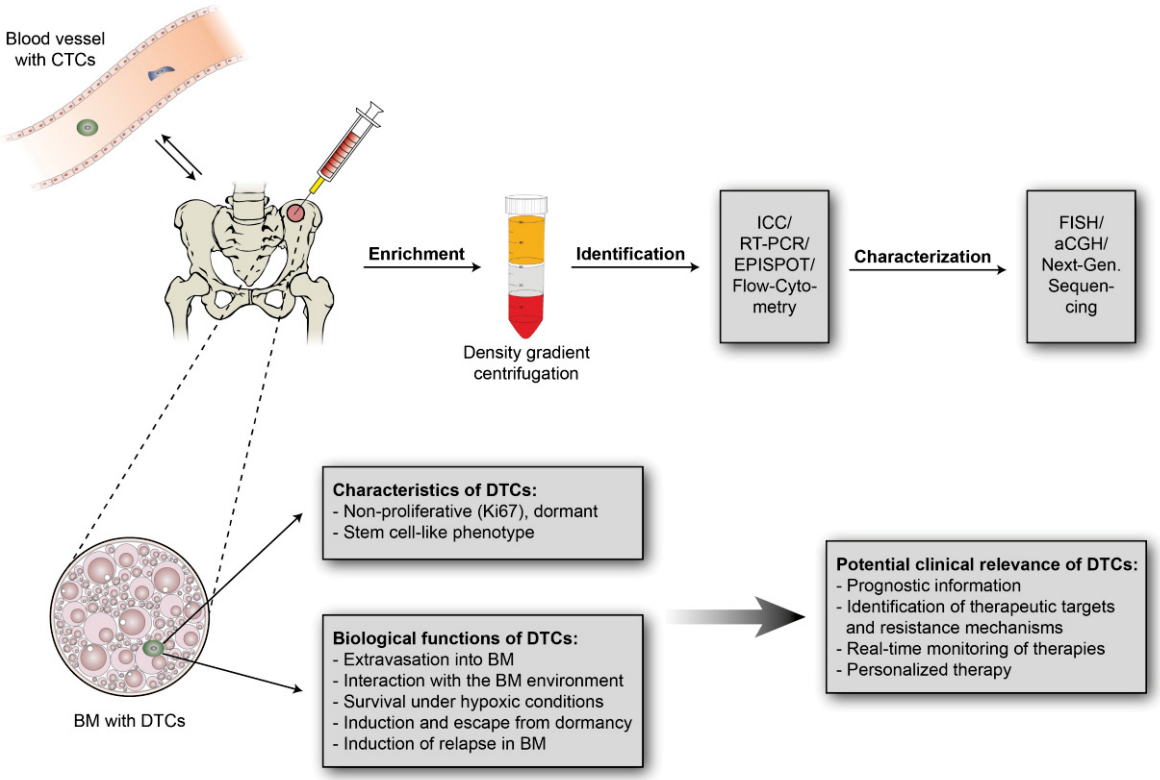

Figure 5: Overview of enrichment, identification, characterization, biology and clinical relevance of disseminated tumor cells (DTCs) in the BM. Primary tumor-derived CTCs disseminate from the blood into the BM (DTCs) but can also re-enter the blood stream and move to other distant organs. aCGH, array comparative genomic hybridization; BM, bone marrow; CTC circulating tumor cell; DTC, disseminated tumor cell; ICC, immunocytochemistry; EPISPOT, EPithelial ImmunoSPOT; FISH Fluorescence *in situ* hybridization RT-PCR, reverse transcriptase-PCR. Modified after [215].

1.6.2 Metastatic latency and cancer dormancy

First, the difference between the terms 'metastatic latency' and ‘dormancy’ needs to be explained. The time period between diagnosis of the primary tumor and the outgrowth of detectable metastasis is designated as 'metastatic latency', while the time that passes until the colonization of the distant organ starts is termed 'dormancy' [215]. Metastatic latency is highly variable between tumor entities. For instance, tumor cells in breast and prostate cancer disseminate quite early and stay/reside for several years or decades at the distant site before building up metastases [215,224,225]. By contrast, distant metastases are detectable within months after primary diagnosis in lung and ovarian cancer patients [3,226]. However, bone manifestation is extremely rare in ovarian cancer (1%-10%), in contrast to breast cancer (50%-85%) and it is supposed that in such a case

the BM is used as a temporary homing site to persist chemotherapy as dormant cells to subsequently induce metastasis in other organs [3,215,227].

Dormancy is characterized by the presence of viable DTCs even many years after removal of the primary tumor without any visible signs of metastasis [48]. There are multiple hints that many cancer patients, which are considered as cured, harbor dormant tumor cells especially within the bone marrow. It is supposed that these cells are dormant as a consequence of a cell cycle arrest [49]. Due to their non-proliferative state, dormant cells are resistant to cytotoxic chemotherapy e.g. DNA-damaging agents (*platinum*) or taxanes which normally affect proliferating/dividing cells [228]. DTCs are mainly detected as single cells, whereas cell clusters that rather would indicate a proliferative state, are very rare [229]. Furthermore, most DTCs are negative for Ki67, a proliferation marker [216]. Accordingly, dormancy might be caused by EMT as it was shown that slow-proliferating or quiescent cells undergo EMT, which indicates that MET is necessary for DTCs to resume proliferation as metastases [77,224].

Various putative factors influencing tumor cell dormancy have been proposed including inefficient angiogenesis, antibody- or T-cell-mediated immune surveillance, lack of proliferative signals, or the activity of metastasis suppressor genes [230]. Since it is the homing site of HSCs, the microenvironment of the BM itself might maintain the dormant state of DTCs [215]. It is anticipated that dormancy-inducing factors such as CXCR4, stem cell factor-1 (SDF-1) and angiopoietin 1 (ANG1), which contribute to HSC quiescence, may also affect DTCs in the BM [231]. Furthermore, it was demonstrated that DTCs co-localize with HSCs in the BM and directly compete with HSCs for habitation of the niche [231]. Balic *et al.* were the first to describe the existence of CD44+ and CD24-/low cells in the BM of breast cancer patients, arguing that these might be cells with a CSC phenotype [232]. However, it is unclear whether this phenotype is caused by an intrinsic CSC capability, by microenvironmental changes or from the composition of the BM itself.

Multiple factors have been proposed that might be involved in escaping dormancy. These can be changes directly restricted to the DTCs such as genetic or epigenetic alterations in genes that control apoptosis or cell proliferation [215]. Additionally, rearrangements in the surrounding microenvironment can directly influence the dormant state by releasing growth factors (e.g. EGF), cytokines (e.g. tumor necrosis factor-α (TNF-α)) or angiogenic factors (e.g. VEGF) [215,224,233]. For instance, TNF-α is released upon chronic inflammation or extensive tissue trauma/damage (wound healing) and leads to the re-activation of dormant cells finally resulting in metastatic outgrowth [215,228]. Apart from that, bone fractures in cancer patients with minimal residual disease might cause

reactivation from dormancy [215]. Similarly, neoangiogenesis was assumed to reactivate (dormant/non-proliferating) DTCs [215,234].

1.6.3 Prognostic relevance of DTCs in ovarian cancer

The majority of clinical studies have provided evidence that the presence of DTCs detected at the time of tumor resection is associated with poor clinical outcome. A decreased PFS/DFS solely [16,209,235,236] or in association with a diminished OS [209,237,238] could be documented various times. One study could demonstrate that DTCs are able to resist chemotherapy [235] and it could be shown that dormant DTCs, which were injected into the mammary fat pad of mice, were capable of developing metastasis in the lung, liver and kidney [239]. However, similar to CTCs, the clinical utility of DTC analyses as a prognostic tool is still under debate [209,216].

1.7 References

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2. Aim of the study

Even though huge efforts have been undertaken to elucidate the biological nature of primary ovarian tumors, the standard therapeutical strategy has not changed decisively in the last 10 years. Additionally many patients who were considered to be cured relapse even short time after resection/treatment of the primary tumor. Possible reasons for the relapse are manifold. Primary tumors are extremely heterogeneous and dispose cells with an increased metastatic potential and insensitivity to chemotherapeutic treatment. As these cells are able to self-renew and differentiate, they are termed cancer stem cells (CSCs). Furthermore the process of EMT plays a major role in cancer progression; it enables tumor cells to detach from the primary tumor and turns them into a more motile phenotype.

Tumor cells that detach from the primary tumor either directly enter the peritoneum or circulate through the blood stream (CTCs) before they disseminate at distant sites (DTCs) where they probably survive chemotherapy and/or induce metastasis. It has been shown that the presence of CTCs and DTCs in ovarian cancer patients has predictive relevance for the patient's OS and/or PFS. CTCs/DTCs with EMT and/or stem-like traits are supposed to play a crucial role in metastatic progression and recurrence.

The aim of this study was to confirm the presence of CTCs and DTCs in ovarian cancer patients as well as to characterize these cells regarding putative stem-like and EMT markers. This included the development of a protocol for a non-invasive detection and molecular characterization of CTCs on the transcriptional level by multiplex-RT-PCR. Furthermore, the presence of DTCs within the BM of ovarian cancer patients before and after implementation of standard chemotherapy and a feasible correlation with the PFS and OS was of particular interest. In order to identify a possible connection between chemotherapy, survival and CSC features, DTCs were also characterized regarding potential stem cell markers.

Revealing unique features of these cells such as a stem-like and/or a more motile phenotype can help to understand the mechanisms underlying the development of resistances and/or therapy failure and to gain deeper insights into potential changes during treatment. The ultimate/long-term aim is to decode potential biomarkers and push forward a more personalized treatment, for instance in form of a real-time monitoring of systemic therapies.

3. Manuscripts

3.1 Gene expression profiling of single circulating tumor cells in ovarian cancer - Establishment of a multi-marker gene panel

Original Research Article in **Molecular Oncology**

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Gene expression profiling of single circulating tumor cells in ovarian cancer – Establishment of a multi-marker gene panel

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ABSTRACT

The presence of circulating tumor cells (CTCs) in the blood of ovarian cancer patients was shown to correlate with decreased overall survival, whereby CTCs with epithelial–mesenchymal-transition (EMT) or stem-like traits are supposed to be involved in metastatic progression and recurrence. Thus, investigating the transcriptional profiles of CTCs might help to identify therapy resistant tumor cells and to overcome treatment failure. For this purpose, we established a multi-marker panel for the molecular characterization of single CTCs, detecting epithelial (EpCAM, Muc-1, CK5/7), EMT (N-cadherin, Vimentin, Snai1/2, CD117, CD146, CD49f) and stem cell (CD44, ALDH1A1, Nanog, SOX2, Notch1/4, Oct4, Lin28) associated transcripts.

First primer specificity and PCR-performance of the multiplex-RT-PCRs were successfully validated on genomic DNA and cDNA isolated from OvCar3 cells. The assay sensitivity of the epithelial panel was evaluated by adding defined numbers of tumor cells into the blood of healthy donors and performing a subsequent immunomagnetic tumor cell enrichment (AdnaTest OvarianCancerSelect), resulting in a 100% concordance for the epithelial markers EpCAM and Muc-1 to the AdnaTest OvarianCancerDetect. Additionally, by

Abbreviations: CTC(s), circulating tumor cell(s); DAPI, 4,6-diamidino-2-phenylindole; DTC(s), disseminated tumor cell(s); DPO, dual priming nucleotides; 6-FAM, 6-carboxyfluorescein; JOE, 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein; FITC, fluorescein-isothiocyanate; TRITC, tetramethyl-rhodamine-isothiocyanate; PDH, pyruvate dehydrogenase; CK, cytokeratin; Muc-1, mucin-1; EpCAM, epithelial cell adhesion molecule; CD117, cluster of differentiation 117 also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit; ALDH1A1, aldehyde dehydrogenase 1 family, member A1; SOX2, SRY (sex determining region Y)-box 2; Oct4, octamer-binding transcription factor 4; FIGO, Fédération Internationale de Gynécologie et d'Obstétrique; MPC, magnetic particle concentrator; Pt, patient; EMT, epithelial–mesenchymal-transition.

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processing blood from ovarian cancer patients, high assay sensitivity could be verified. In blood of healthy donors no signals for epithelial markers were detected, for EMT and stem cell markers, however, signals were obtained mainly originating from leukocytes which calls for single cell analysis.

To that aim by using the ovarian cancer cell line OvCar3, we successfully established a workflow enabling the characterization of single CTCs. It consists of a density gradient-dependent enrichment for nucleated cells, a depletion of CD45-positive cells of hematopoietic origin followed by immunofluorescent labeling of CTCs by EpCAM and Muc-1. Single CTCs are then isolated by micromanipulation and processed for panel gene expression profiling. Finally, fifteen single CTCs from three ovarian cancer patients were analyzed and found to be positive for stem cell (CD44, ALDH1A1, Nanog, Oct4) and EMT markers (N-cadherin, Vimentin, Snai2, CD117, CD146). Albeit, inter-cellular and intra/inter-patient heterogeneity and co-expression of epithelial, mesenchymal and stem cell transcripts on the same CTC was observed.

We have established a robust workflow to perform sensitive single cell panel gene expression analysis without the need of pre-amplification steps. Our data point towards a heterogeneous expression of stem cell and EMT associated transcripts in ovarian cancer CTCs.

1. Introduction

Ovarian cancer is a highly aggressive tumor entity, due to the lack of specific symptoms and screening methods most patients are diagnosed in an advanced stage, which correlates with poor prognosis. Initial debulking surgery aiming at macroscopic complete tumor resection combined with subsequent platinum- and paclitaxel- based chemotherapy is highly effective at inducing remission in patients with advanced ovarian cancer (du Bois et al., 2009). However, more than half of the patients will relapse shortly after an initial response to chemotherapy (Aktas et al., 2011; Fehm et al., 2013; Martin and Schilder, 2009; Rubin et al., 1999). So far, residual postoperative tumor load is one of the most important prognostic factors for the outcome of ovarian cancer (Goodman et al., 2003).

Resistance to platinum-based chemotherapy constitutes a major clinical challenge for ovarian cancer treatment. Several cellular processes can cause platinum resistance, including increased tolerance towards DNA-platinum adducts or enhanced DNA repair capacity of tumor cells (Galluzzi et al., 2012). Moreover, intra-tumor heterogeneity can contribute to chemo-resistance in different ways, which take place on the genomic, transcriptomic, epigenetic and clonal level: i) chemotherapy leads to clonal expansion of intrinsically resistant and pre-existing resistant tumor cells. ii) Chemosensitive tumor cells increasingly convert to a chemo-resistant state and acquire “de novo” therapy resistance. iii) Both mechanisms co-exist (Kuhlmann et al., 2015). Though the link between drug resistance and cellular heterogeneity was initially explored in the context of cancer stem cells, which are present as a small subgroup within the primary tumor (Pribluda et al., 2015; Shah and Landen, 2014). Due to their intrinsic ability to self-renew these CSCs are regarded as the source of metastatic tumor spread and to enhance tumorigenesis and drug-resistance (Dyall et al., 2010; Reya et al., 2001). CSCs have been identified in ovarian cancer cell lines and tissues and their presence has been associated with aggressive tumor behavior (Bapat et al., 2005; Boesch et al., 2014; Hosonuma et al., 2011). CSC heterogeneity may also be increased by the process of epithelial–mesenchymal transition (EMT), which is capable of generating cells with stem cell-like properties from differentiated epithelial cells (Brabletz, 2012; Mani et al., 2008). EMT is a process essential for embryonic development, but also plays a role in tumor progression and metastasis (Thiery, 2002). During EMT epithelial cells of the primary tumor upregulate mesenchymal genes causing them to lose their cell-to-cell adhesions and apicobasal cell polarity, leading to an increase in the cells mobility and invasiveness (Guarino, 2007). It is assumed, that in some cases the combination of EMT and stem cell traits allows tumor cells to escape from the primary tumor, to enter the blood stream and may act as potential metastasis initiating cell.

Several studies have confirmed the prognostic impact of CTCs in ovarian cancer (Abu-Rustum et al., 1999; Aktas et al., 2011; Kuhlmann et al., 2014; Poveda et al., 2011; Zeng et al., 2015; Zhou et al., 2015). Beyond their quantification, a further molecular characterization of CTCs is of high interest to develop CTC-based therapy regimen. Additionally, since CTCs supposedly consist of heterogeneous cell populations with different potentials to survive chemotherapy (Aktas et al., 2011) and to initiate secondary tumors or metastases the use of single cell analysis is required. Only single cell analysis of CTCs allows us to distinguish cells with different expression profiles which give a hint towards the evolution of CTCs during treatment. It will dissect cellular heterogeneity since only a small subset of CTCs from one patient may exhibit the genotype or phenotype responsible for development of therapy resistance. Thereby, single CTC analysis represents a ‘liquid biopsy’ for the selection of an appropriate therapy and for real time monitoring of its effectiveness (Aktas et al., 2009; Barriere et al., 2012; Giordano et al., 2013; Kasimir-Bauer et al., 2012).

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To date, there is no data available showing the expression of stem cell- and EMT associated transcripts expressed in ovarian cancer CTC.[5] However, multiple studies in breast cancer have documented that the expression of stem cell/EMT markers in CTCs is associated with poor prognosis and resistance against chemotherapy (Aktas et al., 2009; Mego et al., 2012) suggesting that their presence should also be investigated in single CTCs from ovarian cancer patients. In order to determine expression of such transcripts in single CTCs, we developed a multiplex PCR approach for genes associated with epithelial [mucin-1 (Muc-1), epithelial cell adhesion molecule (EpCAM), cytokeratin 5&7 (CK5&7)], EMT [N-cadherin, Vimentin, Snai2, cluster of differentiation 117, 146 & 49f (CD117, CD146 & CD49f), Snai1] and stem cell features [cluster of differentiation 44 (CD44), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1), Nanog, SRY (sex determining region Y)-box 2 (SOX2), Notch1, Notch4, Octamer-binding transcription factor 4 (Oct4) and Lin28]. For verification we benchmarked our method to the well-known AdnaTest OvarianCancer.

2. Materials and methods

2.1. Cell line and cell culture

The human ovarian cancer cell line OvCar3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, US) and cultured in RPMI 1640 containing 10% fetal calf serum and 1% (100 U/ml) Penicillin–Streptomycin (Gibco™ by Thermo Fisher Scientific, Waltham MA, US). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. The ovarian carcinoma cell line OvCar3 was used because all markers studied (except for Snai2) are expressed in that cell line.

2.2. Patient samples

The present work is a joint project of the Departments of Gynecology and Obstetrics of the University Hospitals of Duesseldorf and Dresden, Germany. Only patients with histologically confirmed epithelial ovarian cancer were enrolled. Written informed consent was obtained from all participating patients and the study was approved by the local research ethics committees in Duesseldorf (3768) and Dresden (EK 236082012). Clinical patient data are summarized in Table 2 Supplementary Material. Tumors were classified according to the World Health Organization (WHO) classification, grading was done according to Silverberg (Silverberg, 2000) and tumor staging was classified according to FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) (FIGO Committee on Gynecologic Oncology, 2009). The whole study population received primary radical surgery aiming at macroscopic complete tumor resection. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, peritoneal stripping, and pelvic as well as paraaortic lymphadenectomy were performed, where feasible. For bulk analysis a total of 5 ml of peripheral blood was collected in K2 EDTA tubes (Becton Dickinson, Plymouth, UK) and processed within 4 h after phlebotomy. For single cell analysis 7.5 ml of peripheral blood was processed within 8 h after blood collection. To avoid contamination with epithelial skin cells the first blood sample was always discarded.

[5] Pubmed search from 15.01.2016 (January fifteenth two thousand and sixteen), Keywords: ovarian cancer; circulating tumor cells; mesenchymal; EMT; stem cell.

2.3. Blood sample processing

2.3.1. Tumor cell enrichment and isolation for bulk analysis

Cells from ovarian cancer cell lines were dissociated with Accutase (Gibco™ by Thermo Fisher Scientific, Waltham MA, US) and separated with a cellstrainer (Greiner Bio-one, Kremsmünster, Austria) into a single cell suspension. Defined cell numbers (5, 10 and 25) were transferred into 5 ml blood of healthy donors by the MoFlo™ XDP cell sorter (Beckman Coulter, Brea, CA, US). Cells were distinguished based on size and granularity using defined Forward Scatter (FSC) and Side Scatter (SSC) parameters. Cell clumps were excluded by means of SSC-W. All blood samples of patients and healthy donors were subjected to AdnaTest OvarianCancerSelect and/or to the AdnaTest EMT-1/StemCell Select (QIAGEN GmbH, Hilden, Germany) which enable immunomagnetic enrichment of tumor cells positive for epithelial antigens EpCAM and Muc-1. Both tests are using the same CTC enrichment strategy, with a special washing procedure (AdnaWash buffer) included in the AdnaTest EMT-1/StemCell Select to reduce contaminating leukocytes. All experimental steps were performed according to the manufacturer's protocol. In brief, EpCAM- and Muc-1-positive cells were captured and extracted by the Dynal MPC®-S and L magnetic particle concentrators (Invitrogen™ Dynal® by Thermo Fisher Scientific, Waltham, MA). Enriched cells were lysed and RNA was transcribed into cDNA using Sensiscript® Reverse Transcription Kit (QIAGEN GmbH). All spiking experiments were performed at least three times. Blood of healthy donors was examined additionally.

2.3.2. Tumor cell enrichment, identification and isolation for single cell analysis

For spiking experiments 10 dissociated OvCar3 cells were sorted into 7.5 ml of blood from a healthy volunteer as described above. Spiking experiments were performed eight times. These samples and 'control' blood from both ovarian cancer patients and healthy donors was processed as follows. Peripheral blood mononuclear cells (PBMCs) and neutrophils were extracted by Biocoll Separation Solution (Biochrom by Merck, Darmstadt, Germany) gradient centrifugation (density 1.077 g/ml), according to the manufacturer's recommendations. To reduce the number of contaminating leukocytes the PBMC phase was washed for 5 min with 10 ml PBS (Gibco™ by Thermo Fisher Scientific, Waltham MA, US) and CD45 depletion was performed. In brief, 1.5 ml Dynabeads® CD45 (Invitrogen™ by Thermo Fisher Scientific, Waltham, MA, US) were washed two times for 5 min and incubated with the PBMC phase for 30 min whilst rotating. Leukocytes bound to the Dynabeads® CD45 were removed using Dynal MPC®-S (Invitrogen™ Dynal® by Thermo Fisher Scientific, Waltham, MA). Cells of interest were washed twice in 1 ml PBS for 5 min.

To identify CTCs, unfixed and unpermeabilized cells were immunofluorescently stained in suspension with a FITC conjugated mouse monoclonal antibody to EpCAM (clone VU-1D9;

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1:50; Cell Signaling Technology by Merck, Darmstadt, Germany), a FITC conjugated mouse monoclonal antibody to pan-cytokeratin (C11, 1:400, GeneTex, Irvine, CA, US) and 4′,6-Diamidine-2′-phenylindole dihydrochloride ((DAPI) 1 μg/ml Roche, Basel, Switzerland). Leukocytes were identified using a PE-Cy5 conjugated CD45-specific mouse antibody (1:25, Santa Cruz Biotechnology, Dallas, TX, US). Following 2 h incubation in the dark at 4 °C and centrifugation at 450 rpm for 2 min the cell pellet was washed three times with 1 ml PBS for 5 min and processed further.

Single cells were isolated using CellCelector (ALS GmbH, Jena, Germany). This system combines microscopic detection of labeled cells and their automated micromanipulation with a vertical glass capillary fixed to a robotic arm. For microscopy the following set-up was used: Olympus CKX 41; camera system: CCD camera XM10-IR (Olympus, Tokyo, Japan); for analysis ALS CellCelector-Software 3.0, (ALS, Jena, Germany). In detail labeled cell solutions were transferred to a glass slide and cells were allowed to settle. Then CK- and/or EpCAM-positive cells were detected in the FITC channel at a 40× magnification. CK and/or EpCAM-positive cells were selected by the software and additionally recorded in the remaining channels (brightfield (BF), DAPI, and Cy5) at 40× magnification to verify morphology and CD45-negativity of isolated cells. Single cells fullfilling the 'CTC-criteria' i.e. DAPI and CK/EpCAM positivity and CD45 negativity were micromanipulated in DAPI at 40× magnification. Accordingly, selected cells were aspirated with a volume of 20–100 nl using a 30 μm glass capillary. To achieve optimal cell deposition 2–9 μl PBS buffer were taken up into the capillary prior to the picking process. Single cells were deposited into PCR tubes containing 100 μl of lysis buffer of the Dynabeads® mRNA DIRECT Micro Kit (Invitrogen™ Dynal® by Thermo Fisher Scientific, Waltham, MA, US) and stored for up to two weeks at −20 °C until further processing.

Subsequently, oligo (dT) based mRNA isolation was performed for all single cell lysates, according to the manufacturer's protocol with the Dynabeads® mRNA DIRECT Micro Kit (Invitrogen™ Dynal® by Thermo Fisher Scientific, Waltham, MA, US), which is part of the AdnaTest OvarianCancer-Detect. For reverse transcription the Sensiscript® Reverse Transcription Kit (QIAGEN GmbH) was used. The resulting/obtained cDNA served as template for tumor cell detection and characterization by multiplex-PCR.

2.4. Tumor cell detection/analysis of CTCs

2.4.1. AdnaTest OvarianCancerDetect

The AdnaTest OvarianCancerDetect was used to amplify transcripts of the epithelial markers EpCAM, Muc-1 and CA125, whereas the AdnaTest EMT-1/StemCell Detect was employed to analyze ALDH1 expression in a singleplex PCR assay and EMT markers Akt-2, TWIST, PI3Kα in a multiplex-RT-PCR assay. Resulting PCR products were separated using the DNA 1000 LabChips and visualized with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the Expert Software Package (version B.02.07.SI532). Samples analyzed with the AdnaTest OvarianCancerDetect were considered as positive if signals for at least one epithelial marker and the housekeeping transcript β-actin were detected. For the AdnaTest EMT-1/StemCell Detect PCR signals for at least ALDH1 or one EMT product plus a fragment of β-actin had to be present in a positive sample.

2.5. Multiplex-RT-PCR panels

2.5.1. Primer design

Dual priming oligonucleotide primers (DPO) for three independent multiplex-RT-PCR panels were designed using Primer3 Plus software (Rozen and Skaletsky, 2000). In contrast to conventional primers, DPO primers contain two priming sites, which improve binding specificity (Chun et al., 2007). All primers were designed to the 3′ end of each target mRNA to ensure amplification of mRNA degradation products, as well. Forward primers were alternately labeled either with the fluorescent dye 6-Carboxyfluorescein (6-FAM) or JOE (6-Carboxy-4′,5′-Dichloro-2′,7′-Dimethoxyfluorescein), respectively. Additionally, in between one multiplex panel transcript-specific amplicons were designed with at least 20 bp size differences to distinguish them by gel or capillary electrophoresis. Primer sequences, their annealing temperatures and labeling are given in Table1 Supplemented Material. Primers were purchased from Biomers (biomers.net GmbH, Ulm, Germany).

2.5.2. Multiplex-RT-PCR conditions

Multiplex-RT-PCR for all three panels was performed using the KAPA2G Fast Multiplex ReadyMix (Peqlab, Erlangen, Germany) in a final reaction volume of 25 μl. The reaction mixture contained 1 × KAPA2G Fast HotStart DNA Polymerase (1 U/25 μl) KAPA2G Fast HotStart buffer, dNTPs (0.2 mM of each dNTP), $MgCl_2$ (3 mM) and stabilizers. Primers were used in a final concentration of 0.08 μM. For bulk analysis the following amounts of cDNA were used: 4 μl for the epithelial marker panel and 7 μl for stem cell and EMT marker panels. Single cell analysis for all three marker panels (epithelial, EMT and stem cell) was performed with 12.5 μl cDNA. Genomic DNA and cDNA from OvCar3 cells were used as positive control.

The thermal profile used for all multiplex-RT-PCRs was as follows: after an initial denaturation step at 95 °C for 15 min 10 PCR cycles were carried out consisting of denaturation at 95 °C for 45 s, primer annealing/extension at 57 °C for 45 s and elongation for 45 s at 72 °C. Subsequently, annealing/extension temperature was increased for 1 °C per 10 cycles up to 61 °C. Finally, leading to 50 cycles in total. Samples were stored at 4 °C over night or at −20 °C for long-term storage. PCR products were visualized by capillary electrophoresis.

2.6. Capillary electrophoresis

Forward primers were alternately labeled with either 6-FAM or JOE to distinguished PCR products from each other in the same panel. For each sample 15.5 μl of Hi-Di™ Formamide and 1.5 μl of an internal size standard (GeneScan500 ROX, both Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, US) were mixed with 3 μl PCR product (1 μl for controls) and transferred to an ABgene Thermo-Fast 96 PCR Detection Plate (Thermo Fisher Scientific, Waltham, MA, US). For separation of the PCR products an ABI PRISM 3130XL Genetic Analyzer

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(Thermo Fisher Scientific, Waltham, MA) with a capillary of 36 cm length and POP-7 polymer was used. Analysis was performed with the Peak Scanner™ Software Version: 1.0 (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA). A peak was considered as positive if its height excelled 750 rfu (relative fluorescence units). Samples analyzed for epithelial markers by multiplex-RT-PCR were considered as positive for CTCs if signals for at least one epithelial marker and the housekeeping transcript for PDH were detected.

2.7. *Detection and characterization of single leukocytes*

CD45-positive cells were micromanipulated, RNA was isolated and cDNA was synthesized. Then cDNA was analyzed by multiplex-RT-PCR for expression of epithelial, mesenchymal and stem cell transcripts. The remaining 2.5 μl of cDNA were used for CD45-specific PCR using the same thermal profile as applied to all multiplex-RT-PCRs. As positive control reverse transcribed cDNA from 1000 leukocytes was employed. CD45-specific RT-PCR products were visualized with a 2100 Bioanalyzer as described above.

3. Results

3.1. *Establishing three independent multiplex-RT-PCR panels for the detection and characterization of CTCs*

Primer specificity and PCR performance of all three multiplex panels, epithelial (Muc-1, EpCAM, CK5 and CK7), EMT (N-cadherin, Vimentin, Snai2, CD117, CD146, CD49f and Snai1) and stem cell markers (CD44, ALDH1A1, Nanog, SOX2, Notch1, Notch4, Oct4 and Lin28), were successfully established with genomic DNA and cDNA from the ovarian cancer cell line OvCar3. An intron-spanning amplicon for the reference gene pyruvate dehydrogenase (PDH) was included into the epithelial panel to distinguish signals between 'contaminating' genomic DNA (183 bp) and cDNA (100 bp) (Figure 1). With

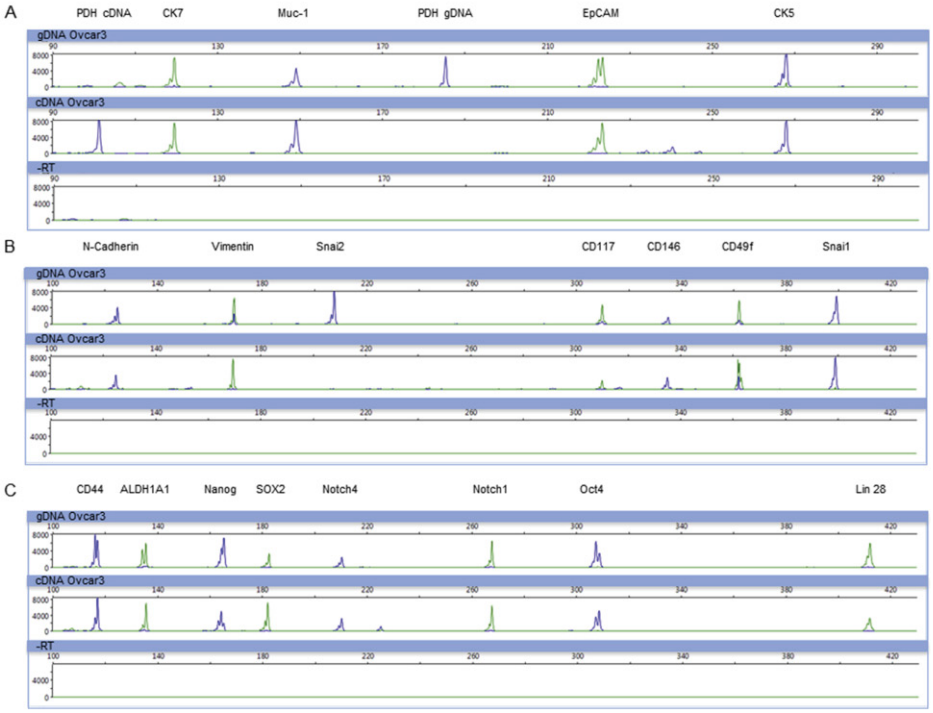

Figure 1 – Multiplex-RT-PCR for the detection of epithelial, EMT and stem cell markers. Depicted are electropherograms of three multiplex-RT-PCR panels after analysis by capillary electrophoresis. For all panels, upper row: gDNA, middle row: cDNA, lower row: -RT. Primer specificity and PCR-performance was successfully validated on genomic DNA and cDNA of OvCar3 cells. A) Epithelial marker panel: CK7 (124 bp), Muc-1 (149 bp), EpCAM (222 bp) and CK5. PDH (100/183 bp) was used as control to distinguish between gDNA and cDNA. B) EMT marker panel: N-cadherin (127 bp), Vimentin (170 bp), Snai2 (208 bp), CD117 (309 bp), CD146 (335 bp), CD49f (363 bp) and Snai1 (402 bp). C) Stem cell marker panel: CD44 (120 bp), ALDH1A1 (139 bp), Nanog (162 bp), SOX2 (185 bp), Notch4 (210 bp), Notch1 (268 bp), Oct4 (310 bp) and Lin28 (413 bp).

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adapted PCR conditions all amplicons were optimized in single PCR and in multiplex reactions (Supplementary Material Figure 1) ensuring an optimal PCR performance without unspecific priming.

3.2. *Validation of multiplex-RT-PCR panels by spiking experiments into blood of healthy donors and blood from ovarian cancer patients*

3.2.1. *Analysis of spiked blood samples*

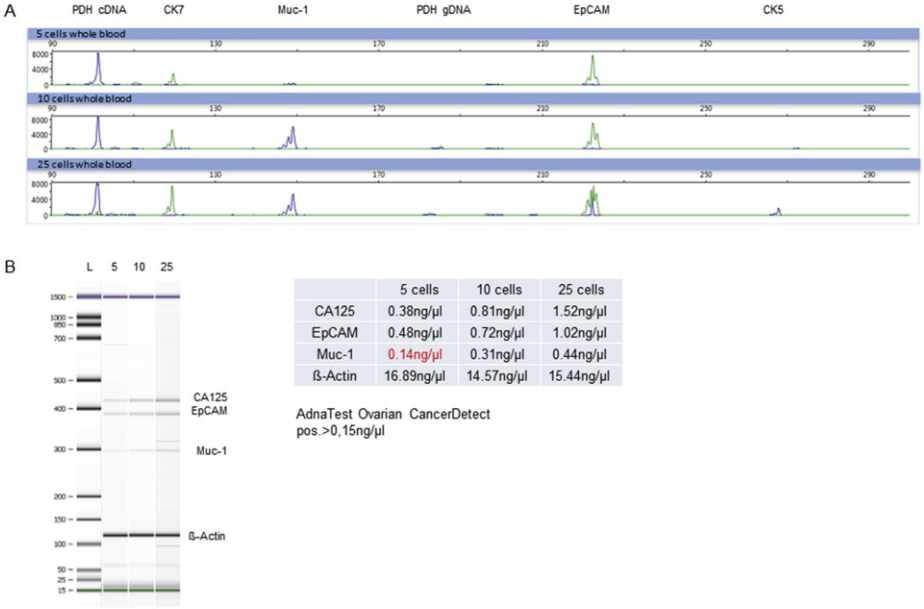
OvCar3 cells express the epithelial cell surface proteins Muc-1 and EpCAM and are thereby suitable for spiking experiments and subsequent enrichment with the AdnaTest OvarianCancer Kit to validate the analytic sensitivity of the epithelial multiplex-PCR panel. Low numbers of OvCar3 cells (5, 10 and 25) were spiked into 5 ml blood of healthy donors and processed with the AdnaTest OvarianCancerSelect. Resulting cDNA of the same sample was used to compare the presence of the epithelial markers EpCAM and Muc-1 by the AdnaTest OvarianCancerDetect and multiplex-RT-PCR. Both tests were 100% concordant (Figure 2).

3.2.2. *Analysis of blood from ovarian cancer patients*

Next, the sensitivity/performance of the epithelial transcript panel was determined with patient blood samples. In total, 10 blood samples from ovarian cancer patients were examined for epithelial markers by the AdnaTest OvarianCancerDetect and the multiplex-RT-PCR. The multiplex-RT-PCR not only confirmed both patients tested positive for CTCs with the AdnaTest OvarianCancerDetect (Table 1 Pt.3 and 4) but also identified a further patient as 'CTC-positive' (Table 1 Pt.1).

3.2.3. *Analysis of blood from healthy donors*

In a next step, blood from healthy donors was assessed as described in 3.2.2. No epithelial markers could be detected with neither AdnaTest OvarianCancerDetect nor multiplex-RT-PCR (Figure 3). However, stem cell- and EMT associated marker signals were obtained by both tests, probably deriving from leukocytes (Supplementary Material Figures 2 and 3). Despite using a washing buffer recommended to reduce co-isolation of leukocytes during the CTC capture phase false positive results were detected.



	5 cells	10 cells	25 cells
CA125	0.38ng/µl	0.81ng/µl	1.52ng/µl
EpCAM	0.48ng/µl	0.72ng/µl	1.02ng/µl
Muc-1	0.14ng/µl	0.31ng/µl	0.44ng/µl
ß-Actin	16.89ng/µl	14.57ng/µl	15.44ng/µl

Figure 2 – Detection of epithelial markers in spiked blood samples after immunomagnetic enrichment with the AdnaTest OvarianCancerSelect. Signals derived from defined numbers of OvCar3 cells (5, 10 and 25) spiked into blood. Positivity for EpCAM and Muc-1 of samples spiked with 5, 10 and 25 OvCar3 cells was 100% concordant to EpCAM and Muc-1 positivity detected by the AdnaTest OvarianCancerDetect A) Visualized are electropherograms of the epithelial multiplex-RT-PCR panel after analysis by capillary electrophoresis. Amplified fragments of the epithelial transcripts CK7 (124 bp), Muc-1 (149 bp), EpCAM (222 bp) and CK5 (265 bp) are shown. PDH is used as control to distinguish between gDNA (183 pb) and cDNA (100 bp). B) Shown are the amplified transcripts Muc-1 (299 bp), EpCAM (395 bp), CA125 (432 bp) and β-Actin (120 bp) by AdnaTest OvarianCancerDetect after electrophoretic separation. Corresponding values (ng/µl) of each transcript are calculated out of the tape thickness and are represented in the table. A signal was considered as positive in case of > 15 ng/µl.

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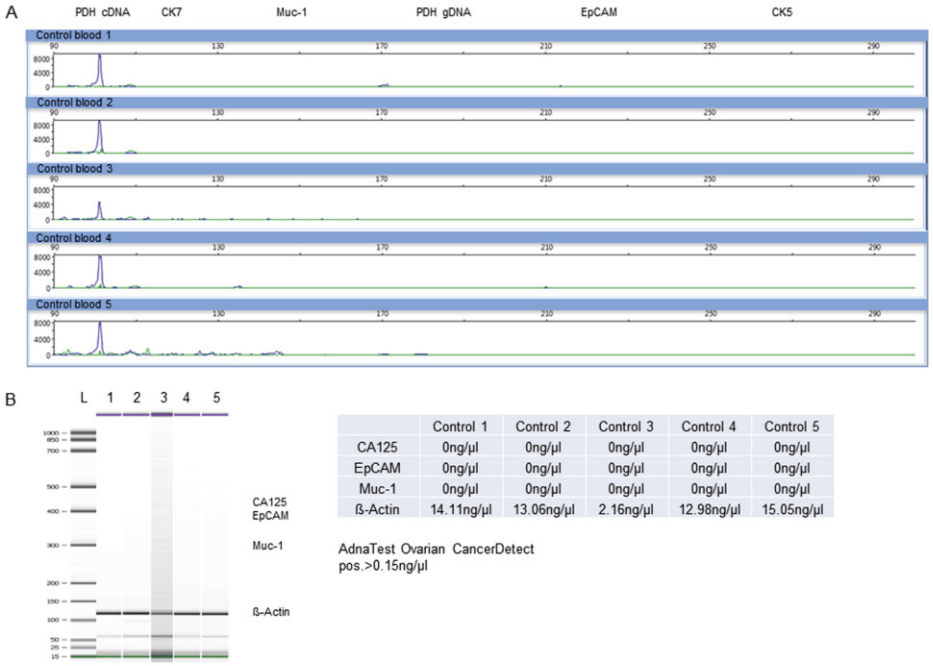
Table 1 – Detection of epithelial markers in 10 ovarian cancer patients, analyzed with multiplex-RT-PCR and AdnaTest OvarianCancerDetect. Samples analyzed by the AdnaTest OvarianCancerDetect were considered as positive for CTCs, if signals for at least one epithelial marker and the housekeeper β-actin were detected. Samples analyzed for epithelial markers by multiplex-RT-PCR were considered as positive for CTCs, if signals for at least one epithelial marker and the housekeeper PDH were detected. Pt. = patient.

Patients	Multiplex RT-PCR epithelial markers	AdnaTest OvarianCancerDetect	Muc-1	EpCAM	CA125	Actin
Pt.1	pos	neg	0 ng/µl	0 ng/µl	0 ng/µl	13.37 ng/µl
Pt.2	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	15.43 ng/µl
Pt.3	pos	pos	0.22 ng/µl	0 ng/µl	0 ng/µl	17.2 ng/µl
Pt.4	pos	pos	0.38 ng/µl	0 ng/µl	0 ng/µl	14.69 ng/µl
Pt.5	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	15.06 ng/µl
Pt.6	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	13.47 ng/µl
Pt.7	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	14.31 ng/µl
Pt.8	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	10.95 ng/µl
Pt.9	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	7.38 ng/µl
Pt.10	neg	neg	0 ng/µl	0 ng/µl	0 ng/µl	10.35 ng/µl

AdnaTest OvarianCancerDetect pos. > 0.15 ng/µl.

3.3. *Panel gene expression profiling of single ovarian cancer cells*

In order to avoid false positive results from non-malignant cells such as leukocytes, a workflow based on spiking experiments of OvCar3 cells into blood of healthy volunteers was established to isolate and characterize single CTCs. It consists of density gradient centrifugation of the blood, depletion of CD45-positive cells, immunofluorescent labeling of CTCs, their micromanipulation, RNA isolation, reverse transcription and analysis by multiplex-RT-PCR (Figure 4A). A tumor cell was classified as CTC and selected for micromanipulation



	Control 1	Control 2	Control 3	Control 4	Control 5
CA125	0ng/µl	0ng/µl	0ng/µl	0ng/µl	0ng/µl
EpCAM	0ng/µl	0ng/µl	0ng/µl	0ng/µl	0ng/µl
Muc-1	0ng/µl	0ng/µl	0ng/µl	0ng/µl	0ng/µl
ß-Actin	14.11ng/µl	13.06ng/µl	2.16ng/µl	12.98ng/µl	15.05ng/µl

Figure 3 – Detection of epithelial markers in blood from healthy donors after immunomagnetic enrichment with the AdnaTest OvarianCancerSelect. For both tests no epithelial markers, only housekeepers (PDH (100 bp) or β-Actin (120 bp)) were detected. A) Visualized are electropherograms of 5 healthy donor samples for the epithelial multiplex-RT-PCR panel after analysis by capillary electrophoresis. B) Displayed are the transcripts of 5 healthy donor samples amplified by the AdnaTest OvarianCancerDetect and separated by capillary electrophoresis. Corresponding values (ng/µl) of each transcript are calculated out of the tape thickness and are represented in the table. A signal was considered as positive in case of > 15 ng/µl.

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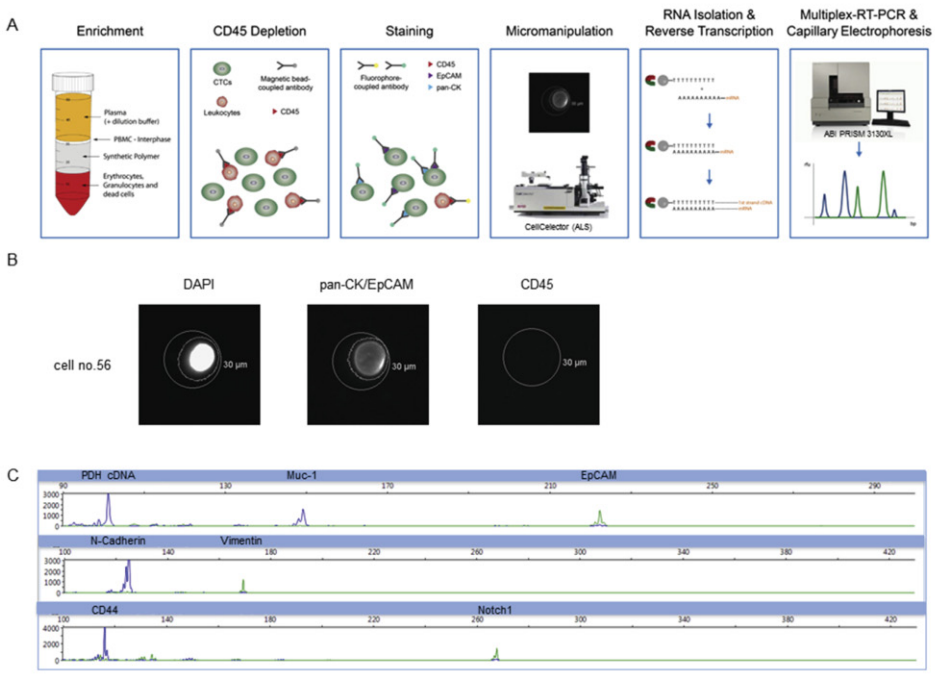

Figure 4 – CTC/tumor cell isolation and analysis on the single cell level. A) Workflow: CTCs were enriched by density gradient centrifugation and CD45-positive cells were depleted. The remaining cells were labeled for EpCAM, pan-CK and CD45 and isolated by micromanipulation. RNA of each single cell was isolated and reverse transcribed into cDNA. Finally, multiplex-RT-PCRs for all 3 panels were performed and PCR products were visualized by capillary electrophoresis. B) Illustration of a single cell (no. 56). A cell was characterized as a CTC in case of DAPI, pan-CK/EpCAM positivity and CD45 negativity as evaluated by microscopy. The outer circle constitutes the capillary, sized with 30 µm. The inner circle is created by the analyzing software indicating a recognized fluorescence signal. B) Electropherograms of epithelial (Muc-1, EpCAM), EMT (N-cadherin, Vimentin) and stem cell markers (CD44) exemplified for one single cell (no. 56). Cells were denoted/designated as CTC-positive in case of positivity for at least one epithelial marker and the housekeeper PDH (cDNA).

when it was positive for DAPI and CK/EpCAM and CD45-negative (Figure 4B).

Regarding multiplex PCR analysis samples were classified as CTC-positive when PCR signals in capillary electrophoresis were present for the housekeeper PDH (cDNA) and at least one epithelial marker and (Figure 4C).

Panel expression profiling of 77 single OvCar3 cells, which were spiked into blood from healthy donors isolated and then processed using selection criteria revealed distinct heterogeneity between single OvCar3 cells especially for mesenchymal and stem cell markers (Figure 5):Twenty-one out of 77 cells simultaneously expressed Muc-1 and EpCAM. Fifteen out of 77 co-expressed CK7, Muc-1 and EpCAM. Only one cell was positive for all four tested epithelial markers (CK7, Muc-1, EpCAM and CK5) (Figure 5 cell no. 7). Only 3 out of 77 cells expressed CK5. N-cadherin was the most prevalent EMT marker (35/77 cells); in 12/35 cells it was co-expressed with vimentin. In 43 of the 77 analyzed cells no stem cell markers were detected. Expression of only CD44 (16/77) or co-expression of Oct4 and Lin28 (4/77) or Notch1 and Notch4 (3/77) was registered. Distribution of all other stem cell markers was non-uniform. Co-expression of epithelial and mesenchymal markers (57/77) as well as mesenchymal and stem cell markers (28/77) or epithelial and stem cell markers (34/77) could be observed frequently. The expression of epithelial, mesenchymal and stem cell markers within one cell was found in 22 of 77 analyzed cells (Figure 5).

Underlining their contaminating potential in bulk analysis single leukocytes were analyzed for all three marker panels and yielded no signals for epithelial markers, while signals for stem cell- and EMT markers were identified (Supplementary Material Figure 4A and B). Their hematopoietic origin was confirmed by detection of CD45 transcript (Supplementary Material Figure 4C Supplemented Data).

3.3.1. *Panel gene expression profiling of single CTCs from ovarian cancer patients*

For single CTC gene expression analysis blood samples from 3 ovarian cancer patients were processed as described in 3.2.2. CTC counts ranged from 4 (Pt. 1 and 3) to 7 (Pt. 2). They were

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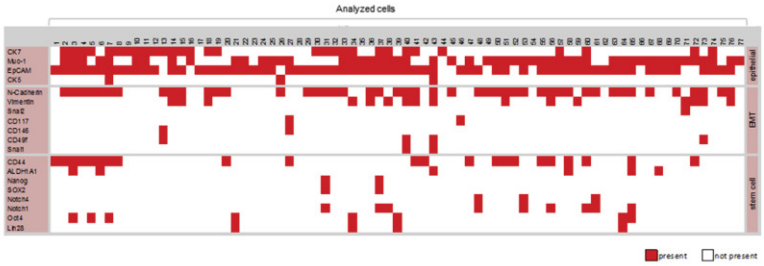

Figure 5 – Expression profiles of 77 single OvCar3 cells spiked into blood of healthy volunteers. Depicted is the expression of epithelial, EMT and stem cell markers (different rows) in 77 single OvCar-3 cells (different columns). A signal was counted as positive reaching a signal intensity of >750 rfu in capillary electrophoresis and is color coded as present (red square). In general a cell was characterized as a tumor cell in case of positivity for PDH (cDNA) and at least one epithelial marker (constituted as present). A distinct intercellular heterogeneity between the 77 analyzed OvCar3 cells originating from one cell line was determined. Not all cells were found to be positive for mesenchymal (EMT) and/or stem cell markers. Furthermore cells with a co-expression of epithelial–mesenchymal (EMT), stem cell-mesenchymal (EMT) and epithelial–mesenchymal (EMT)-stem cell markers could be observed.

isolated via micromanipulation (Figure 6A) and further profiled for their gene expression for epithelial, EMT and stem cell markers (Figure 6B). All CTCs were positive for Muc-1, whereas the presence of other epithelial markers varied. Four of the 15 analyzed CTCs expressed stem cell markers (CD44, ALDH1A1, Nanog and Oct4) and 13 out of 15 were positive for EMT markers (N-cadherin, Vimentin, Snai2, CD117 and CD146) (Figure 6B). Expression profiles differed in CTCs obtained from different patients (inter-patient heterogeneity) and in CTCs isolated from the same patient (intra-patient and

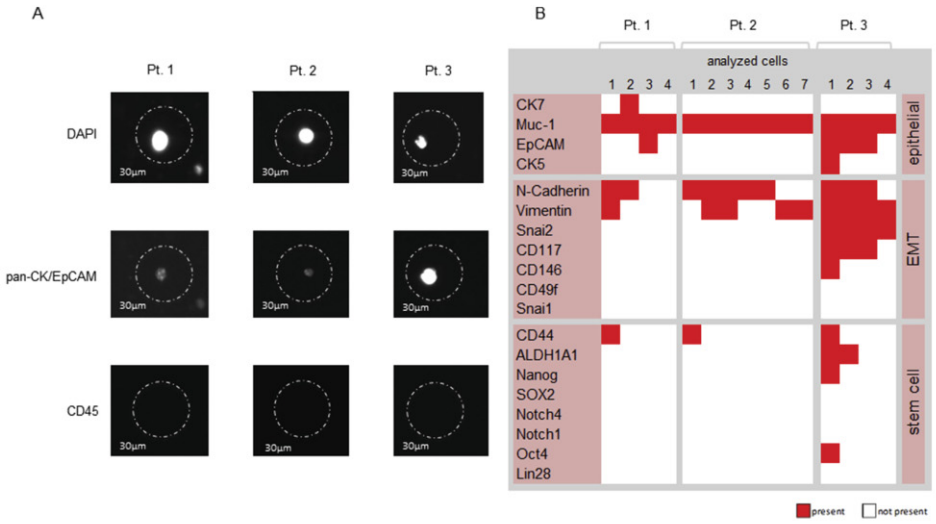

Figure 6 – Single cell expression profiling of CTCs from 3 ovarian cancer patients analyzed by multiplex-RT-PCR for epithelial, EMT and stem cell markers. A) Representative images of single CTCs for each patient after fluorescence microscopy. A cell was characterized as a CTC in case of DAPI, pan-CK/EpCAM positivity and CD45 negativity, as evaluated by microscopy. The circle constitutes the capillary, sized with 30 µm. B) Expression profile of CTCs. A signal was counted as positive reaching a signal intensity of >750 rfu in capillary electrophoresis and is color coded as present (red square). A cell was characterized as a CTC in case of positivity for PDH (cDNA) and at least on epithelial marker after capillary electrophoresis (constituted as present). Expression patterns of all patient samples revealed a ubiquitous expression of Muc-1 in all analyzed CTCs, whereas the expression of additional epithelial markers varied between the cells, even within one patient. In every patient CTCs expressing epithelial, mesenchymal and stem cell markers could be detected even if the amount of CTCs harboring stem cell- and EMT markers was various. Pt. = patient.

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inter-cellular heterogeneity). Furthermore, co-expression of EMT and stem cell markers could be observed.

4. Discussion

Even though huge efforts have been undertaken to elucidate the biological nature of primary tumors and to identify new biomarkers in order to improve the treatment (Serio and Billack, 2011), many patients who were considered to be cured relapse even several years after resection/treatment of the primary tumor (Aktas et al., 2011; Martin and Schilder, 2009). It has already been shown that the presence of CTCs in ovarian cancer patients has predictive and prognostic relevance for the patient's OS and/or PFS (Abu-Rustum et al., 1999; Aktas et al., 2011; Kuhlmann et al., 2014; Poveda et al., 2011; Zeng et al., 2015; Zhou et al., 2015). The pheno- and genotype of disseminated cancer cells have been found to differ from the bulk cell mass of the primary tumor, and CTCs are thought to initiate and establish a secondary tumor at distant sites, thereby worsening the clinical outcome (Polzer et al., 2014). Up to now, therapy strategies for patients diagnosed with ovarian cancer do not consider the phenotype of CTCs. However, revealing unique features of these cells can help to understand the mechanisms underlying the development of resistances and/or therapy failure. CTCs circulate through the bloodstream, they are therefore easily accessible and can be regarded as a 'liquid biopsy' for real time monitoring. Accordingly, characterizing CTCs may support the selection of an appropriate therapy in the near future, thus leading to a more personalized treatment.

Our main goal was to develop a method that combines both the detection and the molecular characterization of single ovarian cancer CTCs. It consists of a CTC enrichment by density gradient centrifugation, CD45 depletion, immunofluorescent labeling, single cell isolation via micromanipulation, reverse transcription and multiplex-RT-PCR analysis.

At first, we successfully validated primer specificity and PCR-performance of all three multiplex panels on genomic-DNA and cDNA of the ovarian cancer cell line OvCar3 (Figure 1). Consequently, we benchmarked the multiplex-RT-PCR approach for the detection of epithelial markers to the commercially available AdnaTest OvarianCancerDetect. Positivity rates for EpCAM and Muc-1 signals for blood samples spiked with different numbers of OvCar3 were 100% concordant with AdnaTest OvarianCancerDetect. Next we confirmed the applicability of our technique for blood samples derived from ovarian cancer patients. As a result the multiplex-RT-PCR not only confirmed both patients tested positive for CTCs by the AdnaTest OvarianCancerDetect but also identified an additional patient as 'CTC-positive', indicating a higher sensitivity/performance of our PCR-based method. Another advantage of our method is, that it only requires half the amount of cDNA as used in the AdnaTest Ovarian Cancer Detect. The reason for this advanced performance most likely lies in the use of DPO primers, which bind with a higher specificity to their target sequences than conventional primers.

So far, analysis of enriched bulk CTC populations is the most common approach to characterize the expression of transcripts (Aktas et al., 2009; Barriere et al., 2012; Kasimir-Bauer et al., 2012) However, this approach may lead to false observations and interpretations due to contamination with non-CTC cells such as leukocytes (Aktas et al., 2009; Sieuwerts et al., 2009). Although they are depleted beforehand, a number of leukocytes usually remain after different CTC enrichment and CD45-based depletion strategies (Sieuwerts et al., 2009). Especially due to their mesenchymal nature, leukocytes interfere with the detection of EMT markers, while incompletely differentiated leukocytes express stem cell markers due to their hematopoietic origin (Bryder et al., 2006). These expression patterns can lead to false positive results. In our experiments this issue was also encountered as blood samples of healthy donors were tested positive for EMT and stem cell transcripts by using both, the AdnaTest OvarianCancerDetect and two multiplex-RT-PCR panels (Supplementary Material Figures 2 and 3). Therefore, we believe that molecular characterization of CTCs is more accurate on the single cell level.

In this study, we describe the development and establishment of a workflow to isolate CTCs from ovarian cancer patients and to perform subsequent gene expression profiling on single cells. This consists of a density gradient-dependent enrichment for nucleated cells, a depletion of CD45-positive cells of hematopoietic origin followed by immunofluorescent labeling of CTCs. Potential CTCs are then isolated by micromanipulation and processed for panel gene expression profiling. We were able to successfully detect and characterize single OvCar3 cells and CTCs isolated from ovarian cancer patients.

We found CTCs expressing both epithelial and mesenchymal genes, which has also been described for CTCs of breast cancer patients on protein level (Kallergi et al., 2011; Yu et al., 2013). An explanation for this phenomenon is that malignant cells escape from the primary tumor and develop a migratory phenotype, which allows them to enter the circulatory system. In addition, disseminated tumor cells persisting in the bone marrow also undergo EMT in order to re-enter the bloodstream. Dynamic changes in the epithelial and mesenchymal proportion of CTCs derived from metastatic breast cancer patients were monitored and an association between treatment resistance and the presence of CTCs with mesenchymal features was detected (Yu et al., 2013). Interestingly, we found both, stem cell and EMT markers co-expressed on single OvCar3 and patient-derived CTCs, supporting the theory that EMT generates a cell population with stem cell-like properties (Mani et al., 2008; Morel et al., 2008).

Similarly to the cellular heterogeneity of the primary tumor, CTCs themselves are likely to consist of a heterogeneous cell population. Our analysis of 77 single OvCar3 cells revealed heterogeneous – gene expression patterns for epithelial, stem cell and EMT markers. We additionally observed this heterogeneity between the blood samples (inter-patient heterogeneity) obtained from our 3 analyzed patients as well as in CTCs of the same patient (intra-patient and inter-cellular heterogeneity).

Our results are in line with previous observations, which has already proposed a heterogeneous cell population with regard to their morphology, molecular characteristics and their metastatic potential (Fehm et al., 2010; Krawczyk et al., 2013; Lianidou et al., 2013) Supporting the hypothesis of different CTC subpopulations, which were selected during platinum-

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based therapy and may be responsible for drug resistance (Bapat et al., 2005; Dyall et al., 2010). However, it has to be noted that all analyzed CTCs expressed Muc-1, in contrast to EpCAM or cytokeratins. This could be due to the small number of patients investigated.

To this day there is no data available on EMT or stem cells markers in ovarian cancer CTCs. Therefore, the aim of this study was to establish a multimarker panel to detect and characterize CTCs. In spite of a small patient cohort, our study offers a unique workflow for the isolation, detection and characterization of single CTCs from ovarian cancer patients.

However, defining which cell is 'THE' CTC with metastasis initiating capacity and the ability to induce recurrence, remains one of the most essential questions in the CTC research field. We believe that sensitive and more accurate assays for molecular characterization of single cells, as described herein, are the first steps to correlate molecular "snapshots" of single ovarian cancer CTCs with clinically relevant phenotypes. For instance, it was already shown that EpCAM-negative CTCs with a defined signature (HER2+/EGFR+/HPSE+/Notch1+) are highly invasive and specifically competent for generating brain and lung metastases in a breast cancer model (Zhang et al., 2013). Therefore, our next goal is to perform a long-time study investigating single CTCs from ovarian cancer patients regarding their expression of epithelial, stem cell and EMT markers before, during and after chemotherapy. By doing this we hope to gain deeper insights into potential changes of their expression profile during treatment, which in the future could help physicians to improve therapeutic intervention.

Limitation of the study: There are multiple techniques available for the quantitative transcriptomic profiling of CTCs. So far, pre-amplification of the mRNA is needed to quantify its quality and to analyze multiple transcripts (Powell et al., 2012; Ting et al., 2015). This approach is prone for technical errors due to an amplification bias.

The multiplex-RT-PCR described herein was designed as a "non-quantitative" approach to simultaneously detect 19 transcripts without prior pre-amplification in order to provide a time- and cost-efficient screening tool for longitudinal molecular characterization of CTCs e.g. during chemotherapies. For subsequent quantification of identified therapy-relevant candidate transcripts RT-qPCR can be applied by using the same primers.

So far our workflow exclusively focuses on epithelial-associated markers to enrich for CTCs, which only allows characterization of a restricted set of CTCs. However, we would like to refrain from naming those cells of interest "epithelial CTCs", since we believe that CTCs captured with EpCAM/CK antibodies do not necessarily exhibit a completely epithelial phenotype. In this context, we and others have already observed co-expression of mesenchymal/epithelial markers in CTCs enriched by epithelial epitopes, which may represent an "intermediate state" (Aktas et al., 2009; Kasimir-Bauer et al., 2012; Schneck et al., 2015). This CTC subpopulation expresses epithelial-associated surface antigens, which makes them accessible for enrichment by EpCAM as well as CK. Nevertheless they may already express EMT or stem cell associated genes which indicate the beginning of EMT. This is in accordance with the hypothesis that EMT stem or epithelial associated CTC traits represent rather a "continuum", with a high degree of plasticity than a sharply defined phenotypes (Scheel and Weinberg, 2012; Yu et al., 2013).

However, the enrichment strategy used herein is exemplarily and our multiplex assay for CTC detection is designed to allow combinations with any other enrichment strategy (such as selection marker independent filtration or microfluidic separation). The only prerequisite is that cells remain intact during isolation, which excludes e.g. cell permeabilization. In future studies we plan to apply our multiplex panel also downstream of other enrichment strategies, e.g. for the molecular characterization of EpCAM-negative CTC. We recently published a detection and isolation strategy for such kind of CTCs derived from breast cancer patients (Schneck et al., 2015) and we plan to adapt this method to ovarian cancer.

5. Conclusion

Taken together, we developed a workflow for the detection and gene expression profiling of single CTCs from ovarian cancer patients. Our multiplex-RT-PCR is inexpensive, versatile and applicable to upstream CTC enrichment strategies, working in a non-invasive manner to the cells. This multiplex assay allows the detection of 19 epithelial-, EMT- or tumor stem cell-associated markers of single cells without pre-amplification of transcripts or co-isolation of contaminating non-malignant cells. Amplicons of all 3 panels can be combined and panels can be extended underscoring the usability and versatility of our technique.

Conflict of interest

All authors declare that they have no conflicts of interest regarding the contents of this manuscript.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.04.002.

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Supplementary Material Table 1

Primer sequences, amplicon sizes and labeling for multiplex-RT-PCR products.

Primer name	Sequence (5` -> 3`)	Product size (bp)	Labeling
Epithelial/control			
PDH_fw	GGTATGGATGAGGAGCTGGA	100/183bp	6-FAM
PDH_rev	CTTCCACAGCCCTCGACTAA		
CK7_fw	GGGCTCCTGAAGGCTTATTC55555GGACCGCATC	124bp	JOE
CK7_rev	GGGTGGGAATCTTCTTGTGA55555GGTGGTGGCT		
Muc-1_fw	CCATTCCACTCCACTCAGGT55555AGGGCCAGAG	149bp	6-FAM
Muc-1_rev	CCACATGAGGCTTCCACACAC55555AGTGTCCGAG		
EpCAM_fw	TGTTTGGTGATGAAGGCAGA55555ATGGCTCAAA	222bp	JOE
EpCAM_rev	CACTCGCTCAGAGCAGGTTA55555GTGTCCTTGT		
CK5_fw	CAAGCAGTGTTTCCTCTGGA55555CAGTGGCAGT	265bp	6-FAM
CK5_rev	GAGGAGGTGGTGGAGACAAA55555CGCTGGAGCT		
EMT			
N-Cadherin_fw	TGACACTGTGGAGCCTGATG55555AAGCCTGTGG	127bp	6-FAM
N-Cadherin_rev	GAAGTCCCCAATGTCTCCAG55555GGGGCTGCAG		
Vimentin_fw	GAGAACTTTGCCGTTGAAGC55555ACTACCAAGA	170bp	JOE
Vimentin_rev	TCCAGCAGCTTCCTGTAGGT55555TCTCAATGTC		
Slug_fw	GAGCATACAGCCCCATCACT55555GACTACCGCT	208bp	6-FAM
Slug_rev	GGGTCTGAAAGCTTGGACTG55555TTTCCTCTTC		
CD117_fw	GTGGGAAAACACTGCCATCT55555TGGATTCTTA	309bp	JOE
CD117 _rev	TAGGGACTGATGCCTTCCAC55555CTGCACCACT		
CD146_fw	CTCATGTTGAAGTGCGCTGT55555CCCGCTCCGG	335bp	6-FAM
CD146_rev	ATAGGTGCCAACCACTACGC55555TAATTTTTTT		
CD49f_fw	GCATGGGAGGTCATCACTTT55555AAATTATTTA	363bp	JOE
Cd49f_rev	TTGGATCCACCATAGGCATT55555TTTCTTTCAA		
Snai1_fw	GCTCCACAAGCACCAAGAGT55555TGCTCAGGAT	402bp	6-FAM
Snai1_rev	GGAGCTTCCCAGTGAGTCTG55555CTTTGTCCTG		
Stem cell			
CD44_fw	GTGTGGGCAGAAGAAAAAGC55555ATCAACAGTG	120bp	6-FAM
CD44_rev	TTGTTCACCAAATGCACCAT55555GAGACTTGCT		
ALDH1A1_fw	GGACCAGTGCAGCAAATCAT55555TTAAATCTTT	139bp	JOE
ALDH1A1_rev	CCTGCAGAGCAGAGGAGATT55555GGCTTTATCA		
Nanog_fw	ATGCAGTTCCAGCCAAATTC55555CCAGTGACTT	162bp	6-FAM
Nanog_rev	TCACACGTCTTCAGGTTGCATGT55555GAGTAGTTTA		
SOX2_fw	ACCTCTTCCTCCCACTCCAG55555CCTGCCAGGC	185bp	JOE
SOX2_rev	ATGTGTGAGAGGGGCAGTGT55555TAATGGCCGT		
Notch4_fw	CTGCGATAATGCGAGGAAGA55555AGTGGCTGCC	210bp	6-FAM
Notch4_rev	GGTTTATGGGCATTTCTTGG55555TGGGGGACCA		
Notch1_fw	TCCACCAGTTTGAATGGTCA55555AGTGGCTGTC	268bp	JOE
Notch1_rev	AGTTTTGTGGCTGCACCTG55555TCTGCACCAG		
OCT4_fw	AGCGATCAAGCAGCGACTAT55555ACGAGAGGAT	310bp	6-FAM
OCT4_rev	CAAAAACCCTGGCACAAACT55555TTTTCTTTCC		
Lin28_ fw	CATTCAGTGCCAGGGAGAAT55555CAGGTTGGTT	413bp	JOE
Lin 28_ rev	TAAAGGACTGGCAACCCAAC55555GCATTTTCGT		
Leukocytes			
CD45_fw	AGCACCTACCCTGCTCAGAA55555AAGTAAAGAA	230bp	none
CD45_rev	TTTAAAGCTGGACTTGCAGGA55555GACAGAATGT		

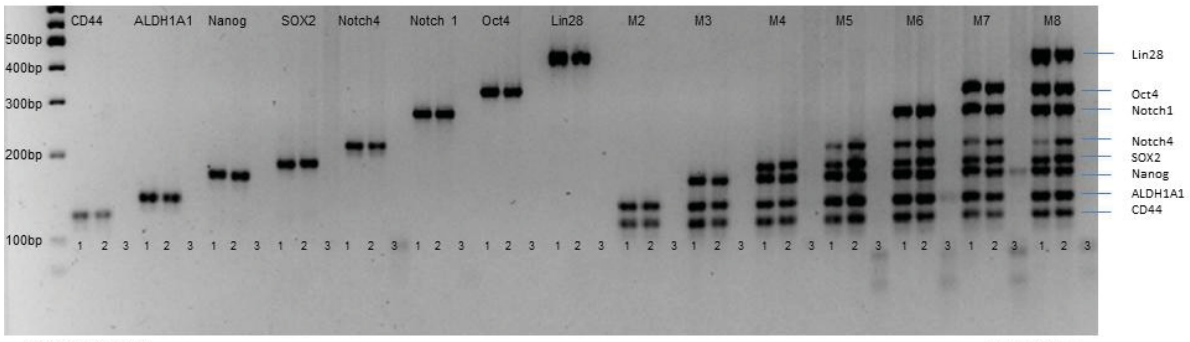
Supplementary Material Table 2

Patient characteristics (Pt. = patient)

	bulk analysis										single cell analysis		
	Pt.1	Pt.2	Pt.3	Pt.4	Pt.5	Pt.6	Pt.7	Pt.8	Pt.9	Pt.10	Pt.1	Pt.2	Pt.3
Age at diagnosis	52	47	66	80	57	36	78	73	56	71	55	69	77
primary diagnosis	n.a.	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	yes	no
FIGO-stage	3a	3b o. c	4	3c	3b	1a	1a	n.a.	1a	3c	1c	1a	4
pN	1	x	1	1	0	x	0	n.a.	0	1	0	0	x
Grading	low grade	low grade	high grade	high grade	high grade	low grade	high grade	n.a.	low grade	high grade	high grade	high grade	high grade
Histology	serous	stromal sarkoma	serous	serous	serous	mucinous	serous	n.a.	mucinous	serous	serous papillary	serous	serous
n.a. = not available													

Supplementary Material Figure 1

Establishment of a multiplex-RT-PCR exemplified for the detection of stem cell markers. All amplicons were tested in single RT-PCR reactions and multiplex-RT-PCRs for up to 8 markers. PCR products were visualized by gel electrophoresis on an agarose gel (3%).

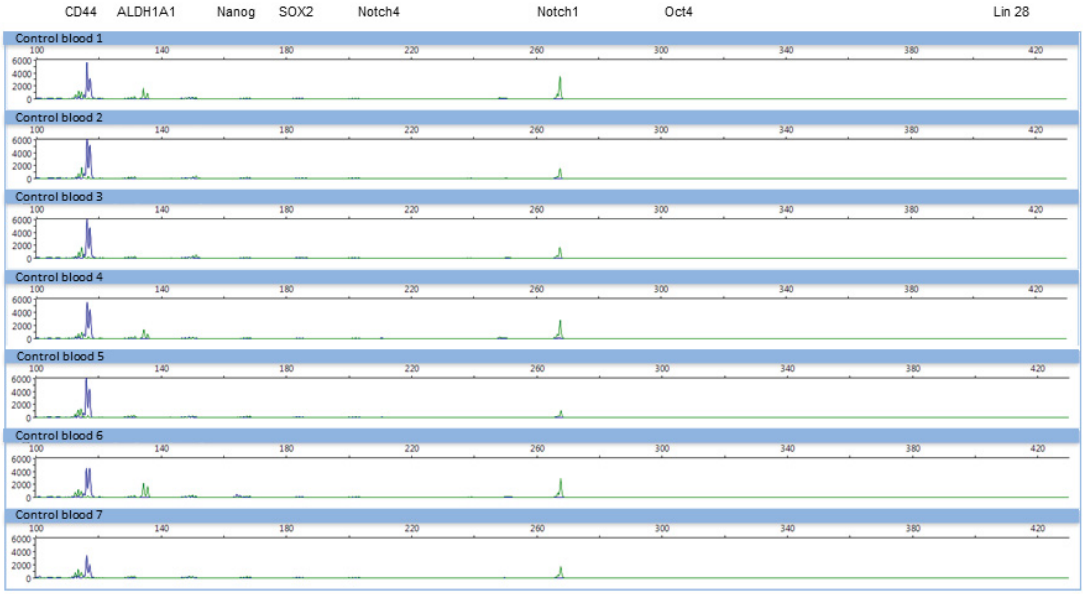


M2= CD44 + ALDH1A1
M3= CD44 + ALDH1A1 + Nanog
M4= CD44 + ALDH1A1 + Nanog + SOX2
M5 =CD44 + ALDH1A1 + Nanog + SOX2 + Notch 4
M6= CD44 + ALDH1A1 + Nanog + SOX2 + Notch 4 + Notch 1
M7= CD44 + ALDH1A1 + Nanog + SOX2 + Notch 4 + Notch 1 + Oct 4
M8= CD44 + ALDH1A1 + Nanog + SOX2 + Notch 4 + Notch 1 + Oct 4 + Lin28

1) gDNA OvCar3
2) cDNA OvCar3
3) H_2O

Supplementary Material Figure 2

Detection of stem cell markers in blood from healthy donors after immunomagnetic enrichment with the AdnaTest EMT-1/StemCell Select. A) Visualized are electropherograms of the stem cell multiplex-RT-PCR panel after analysis by capillary electrophoresis. Amplified fragments of the stem cell transcripts CD44 (120 bp), ALDH1A1 (139 bp) and Notch1 (268 bp) are shown. In all control blood samples CD44 and Notch1 could be detected, whereas ALDH1A1 was identified in 3 out of 7. B) Noted is the amount of the amplified transcript ALDH1 in ng/µl after AdnaTest EMT-1/StemCell Select, which was detected in 2/7 analyzed blood samples from healthy donors. The CTC enriched fraction still contained leucocytes, which interfered with our stem cell panel, as well as with those of the AdnaTest EMT-1/Stem Cell Detect in blood of healthy donors.

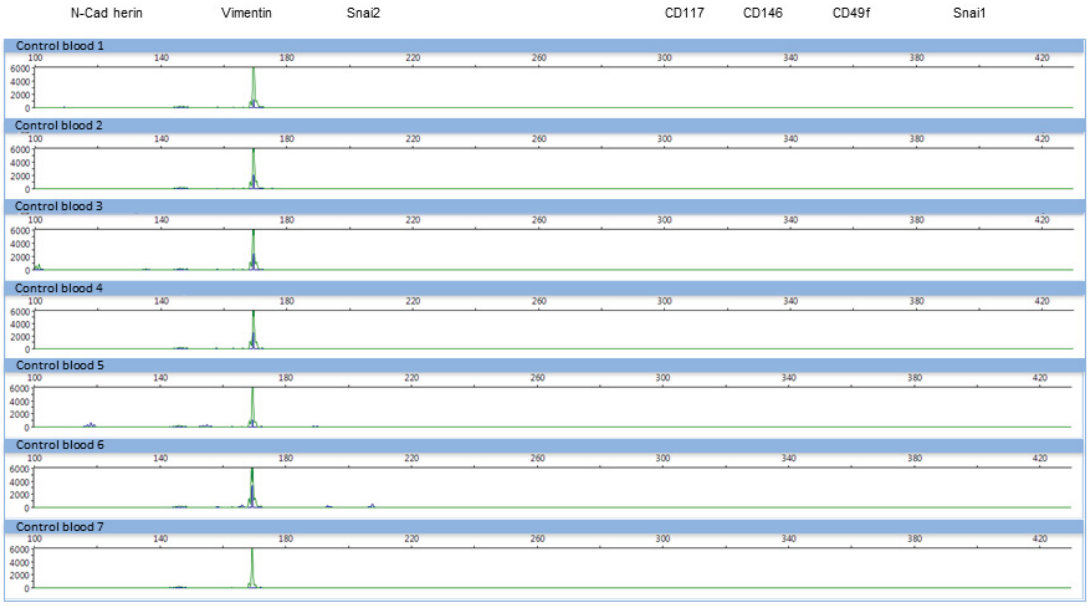


	ALDH1
Control 1	0.00 ng/µl
Control 2	0.00 ng/µl
Control 3	0.00 ng/µl
Control 4	0.16 ng/µl
Control 5	0.00 ng/µl
Control 6	0.26 ng/µl
Control 7	0.00 ng/µl

AdnaTest EMT-1/StemCell Detect
pos.>0,15ng/µl

Supplementary Material Figure 3

Detection of EMT markers in blood from healthy donors after immunomagnetic enrichment with the AdnaTest EMT-1/StemCell Select. A) Visualized are electropherograms of the EMT multiplex-PCR panel after analysis by capillary electrophoresis. The amplified fragment of Vimentin (170 bp) was detected in all blood samples. B) Noted is the amount of the amplified transcripts PIK3CA, Akt2, TWIST1 and β-Actin in ng/µl after AdnaTest EMT-1/StemCell Select. In 3 out of 7 analyzed blood samples Akt2 was detected as positive. The CTC enriched fraction still contained leucocytes, which interfered with our EMT-panel, as well as with those of the AdnaTest EMT-1/Stem Cell Detect in blood of healthy donors.

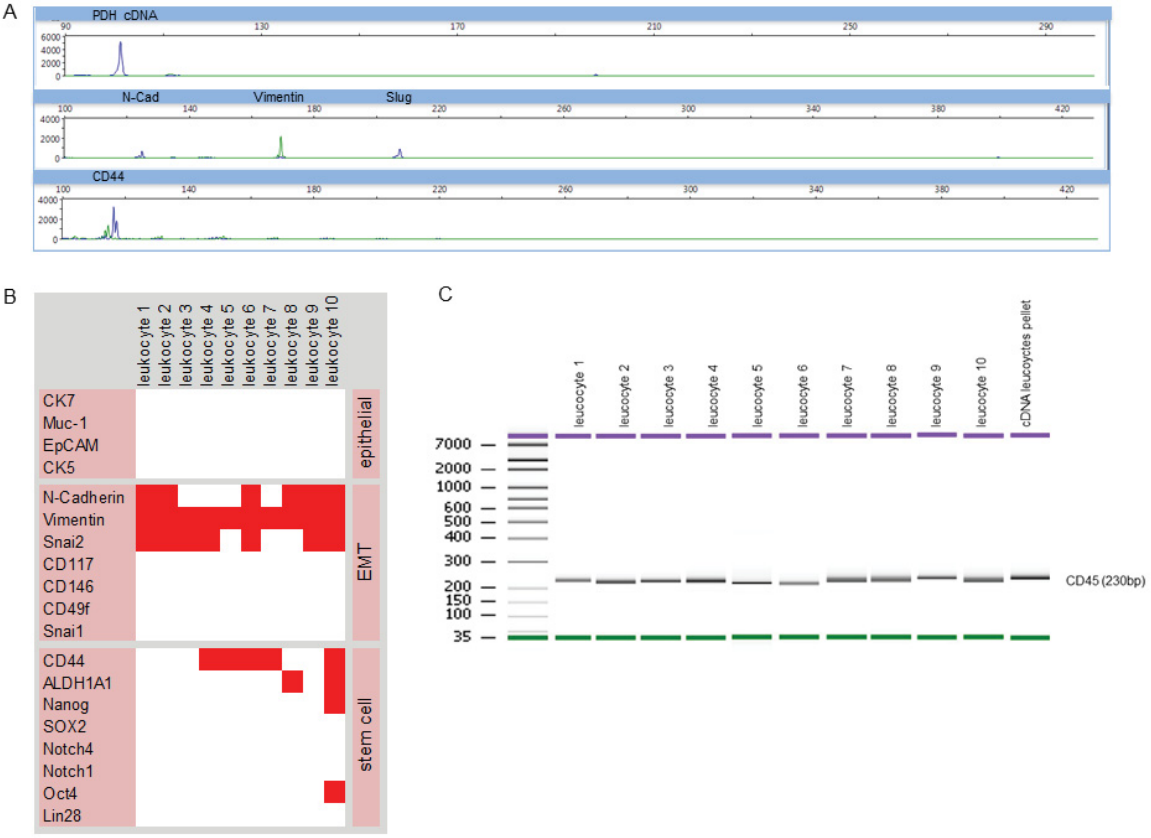


	PIK3CA	Akt-2	TWIST1	ß-Actin
Control 1	0.14ng/µl	0.00ng/µl	0.00ng/µl	5.87ng/µl
Control 2	0.00ng/µl	0.51ng/µl	0.00ng/µl	4.94ng/µl
Control 3	0.00ng/µl	0.11ng/µl	0.00ng/µl	7.27ng/µl
Control 4	0.00ng/µl	0.52ng/µl	0.00ng/µl	5.74ng/µl
Control 5	0.00ng/µl	0.10ng/µl	0.00ng/µl	3.56ng/µl
Control 6	0.20ng/µl	0.69ng/µl	0.00ng/µl	8.99ng/µl
Control 7	0.00ng/µl	0.10ng/µl	0.00ng/µl	2.46ng/µl

AdnaTest EMT-1/StemCell Detect pos.>0,25ng/µl

Supplementary Material Figure 4

Expression profiling of single leukocytes analyzed by multiplex-RT-PCR for epithelial, EMT and stem cell markers A) Electropherograms of epithelial, EMT and stem cell-markers exemplified for a single leukocyte. No epithelial markers could be observed, whereas the stem cell marker CD44 and the EMT markers N-cadherin, Vimentin and Snai2 were detected. B) Expression profile of 10 single leukocytes analyzed by multiplex-RT-PCR for epithelial, EMT and stem cell markers. In none of the analyzed leukocytes epithelial markers could be observed, whereas EMT markers were detected in all cases, and stem cell markers in 6 out of 10 cells. C) Detection of CD45 on single leukocytes. CD45 PCR fragments from single leukocytes were visualized with the Bioanalyzer 2100 (Agilent Technologies) and cells could be identified as leukocytes.



3.2 Pooled Analysis of the Prognostic Relevance of Disseminated Tumor Cells in the Bone Marrow of Patients With Ovarian Cancer

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ORIGINAL STUDY

Pooled Analysis of the Prognostic Relevance of Disseminated Tumor Cells in the Bone Marrow of Patients With Ovarian Cancer

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Objective: Detection of disseminated tumor cells (DTCs) in the bone marrow (BM) of patients with breast cancer is associated with poor outcomes. Recent studies demonstrated that DTCs may serve as a prognostic factor in ovarian cancer. The aim of this 3-center study was to evaluate the impact of BM status on survival in a large cohort of patients with ovarian cancer.

Materials and Methods: Four hundred ninety-five patients with primary ovarian cancer were included in this 3-center prospective study. Bone marrow aspirates were collected intraoperatively from the iliac crest. Disseminated tumor cells were identified by antibody staining and by cytomorphology. Clinical outcome was correlated with the presence of DTCs.

Results: Disseminated tumor cells were detected in 27% of all BM aspirates. The number of cytokeratin-positive cells ranged from 1 to 42 per 2×10^6 mononuclear cells. Disseminated tumor cell status did correlate with histologic subtype but not with any of the other established clinicopathologic factors. The overall survival was significantly shorter among DTC-positive patients compared to DTC-negative patients (51 months; 95% confidence interval, 37–65 months vs 33 months; 95% confidence interval, 23–43 months; $P = 0.023$). In the multivariate analysis, BM status, International Federation of Gynecology and Obstetrics stage, nodal status, resection status, and age were independent predictors of reduced overall survival, whereas only BM status, International Federation of Gynecology and Obstetrics stage, and resection status independently predicted progression-free survival.

Conclusions: Tumor cell dissemination into the BM is a common phenomenon in ovarian cancer. Disseminated tumor cell detection has the potential to become an important biomarker for prognostication and disease monitoring in patients with ovarian cancer.

Key Words: Ovarian cancer, Disseminated tumor cells, Overall survival, Progression-free survival, Bone marrow

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Ovarian cancer is currently the fifth leading cause of cancer death of women in Europe and the United States.[1] The prognosis of patients with ovarian cancer is limited owing to the lack of early symptoms and a high rate of recurrence. The disease is diagnosed in most patients at an advanced stage, and although the initial response to chemotherapy is generally good, a significant proportion of patients will have a relapse despite optimal treatment based on current guidelines.[2] The identification of new biomarkers, reflecting current disease status and tumor activity, could optimize prediction and monitoring of oncologic treatment and provide insights into the biology of ovarian cancer. In this regard, recent studies have increasingly focused on disseminated tumor cells (DTCs) in the bone marrow (BM).

The presence of DTCs is a common phenomenon in solid tumors of epithelial origin. For breast cancer, DTC detection has been demonstrated to be a strong independent prognostic factor (level I evidence).[3] Available data support the notion that hematogenous tumor cell dissemination may be clinically relevant in ovarian cancer as well.[4–8] Detection rates of DTC, as a surrogate parameter for occult hematogenous spread, vary between 30% and 50% of patients with primary ovarian cancer. Interestingly, ovarian metastases to the bone are only rarely observed.[5,9,10] Possibly, BM acts as a secondary "homing site" in these patients where tumor cells are able to persist and may subsequently cause a relapse.

The aim of the present 3-center study was to prospectively evaluate the impact of BM status on clinical outcome in a group of 495 patients treated for ovarian cancer at 3 comprehensive cancer centers (University Hospital Tuebingen, Germany; University Hospital Essen, Germany; and University Hospital Munich, Germany).

MATERIALS AND METHODS

This 3-center analysis was performed at the Department of Obstetrics and Gynecology, University Hospital Tuebingen, Germany; the Department of Gynecology and Obstetrics, University Hospital Essen, Germany; and the Department of Gynecology and Obstetrics, University Hospital Munich, Germany. A total of 495 patients with ovarian cancer patients (International Federation of Gynecology and Obstetrics [FIGO] stages I–IV) treated between January 1994 to November 2010 in these centers were included in the analyses (Tuebingen, 229 patients [46%]; Essen, 148 patients [30%]; and Munich, 118 patients [24%]). The patients' characteristics at the time of diagnosis are shown in Table 1. All specimens were obtained after written informed consent. Tissue sampling and analysis of data were approved by the local ethic committee (114/2006A, 05-2870). Treatment of ovarian cancer and follow-up examinations were performed according to current treatment guidelines. Relapse was confirmed by physical examination, computed tomographic scan, x-ray, ultrasound, tumor marker, and/or relaparotomy depending on localization of recurrence.

Preparation of Slides

Detection of DTCs was performed as described in detail previously for all 3 centers.[4] Ten to 20-mL BM was aspirated intraoperatively from the iliac crest of both sides into syringes containing heparin anticoagulant under general anesthesia using the Jamshidi technique. Bone marrow samples were processed within 24 hours. Tumor cell isolation and detection were performed based on the recommendations for standardized tumor cell detection.[11] Briefly, samples were separated by density centrifugation using Ficoll (density, 1077 g/mL; Biochrom, Germany). Mononuclear cells (MNCs) were collected from the interphase layer, spun down onto a glass slide (10^6 MNCs per spot; Hettich cytocentrifuge, Tuttlingen, Germany) and air-dried overnight at room temperature.

TABLE 1. Incidence of DTC in patients with ovarian cancer based on diagnosis and clinical-pathological factors

	No. Patients (N = 495)	DTC Positive, n (%)	*P*
Total	495	134 (27)	
FIGO stage			0.459
I	87	27 (31)	
II	33	12 (36)	
III–IV	342	93 (27)	
Nodal status			0.061
N0	211	70 (33)	
N1	209	52 (25)	
Grading			0.107
G1	41	6 (15)	
G2	184	57 (31)	
G3	223	65 (29)	
Histologic type			0.026
Serous high-grade	313	88 (28)	
Serous low-grade	30	2 (7)	
Mucinous	19	9 (47)	
Endometrioid	29	8 (28)	
Clear cell	10	5 (50)	
Other	11	3 (27)	
Resection status			0.632
No remaining tumor	239	73 (30)	
Tumor rest	190	54 (28)	
Age, yrs			0.116
0–29	12	2 (17)	
30–49	108	27 (25)	
50–69	273	68 (25)	
70–89	102	37 (36)	

Staining of Slides and DTC Identification

For detection of cytokeratin (CK)-positive tumor cells, slides were fixed in 4% neutral buffered formalin for 10 minutes and rinsed in phosphate-buffered saline. Automatic immunostaining was performed on the Dako Autostainer

TABLE 2. Survival analysis of 456 patients depending on BM status

	No. Patients (N = 456)	Deaths, n (%)	Relapses, n (%)
Total		196 (43)	198 (43)
DTC status			
DTC positive	125	64 (51)	60 (48)
DTC negative	331	132 (40)	138 (42)

Relapse is defined as either local recurrence or distant metastasis (median follow-up: 46 months).

using the monoclonal mouse A45-B/B3 antibody (Micromet, Munich, Germany) and the DAKO-APAAP detection kit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. The A45-B/B3 antibody is directed against a common epitope of CK polypeptides, including the CK heterodimers 8/18 and 8/19. The A45-B/B3 antibody is the most extensively studied antibody for DTC detection in ovarian cancer to date.[4,5,9,12] Reproducibility of DTC detection by A45-B/B3 antibody has been tested by using ovarian cancer cell line SKOV3.[12] In our analysis, for reasons of feasibility in the clinical laboratory routine the malignant breast cancer cell line MCF-7 was used as a positive control. For each patient, 2×10^6 cells were analyzed on 2 slides. All slides were evaluated by 2 independent cytologists. In case nonconcordant results were obtained, the slides were evaluated by a third investigator to obtain consensus. Identification of tumor cells was performed according to the ISHAGE evaluation criteria and the DTC consensus statements.

Statistical Analysis

The $\chi 2$ test and the Fisher exact test were used to evaluate the relationship between circulating tumor cells (CTCs) and clinicopathological factors. For the survival analysis, we considered in separate analyses the following primary end points: (1) death and (2) relapse, defined as distant or local disease recurrence, or both. Survival intervals were measured from the time of BM aspiration to the time of death or of the first clinical, histological, or radiographic diagnosis of relapse. We constructed Kaplan-Meier curves and used the log-rank test to assess the univariate significance of the parameters. The effects of multiple variables on survival were evaluated by a Cox proportional-hazards regression model. All reported P values are 2-sided. The initial model included BM status, age at diagnosis, FIGO stage, nodal status, resection status, and grading. Subjects with missing values were excluded from modeling. Statistical analysis was performed by SPSS version 17.0 (SPSS Inc, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

Incidence of DTC

Bone marrow aspirates were obtained intraoperatively from 495 patients with primary ovarian cancer. Most (74%) of the patients were at FIGO stages III to IV, 19% at FIGO stage I, and 7% at FIGO stage II. The mean age was 58 years (range, 18–88 years). Disseminated tumor cells were detected in 134 (27%) of 495 BM aspirates. The number of CK-positive cells ranged from 1 to 42 per 2×10^6 mononuclear cells. Bone marrow status did not correlate with age ($P = 0.116$), FIGO stage ($P = 0.459$), lymph node status ($P = 0.061$), histopathologic grading ($P = 0.107$), or resection status ($P = 0.632$; Table 1). The lowest prevalence of positive BM status was observed in patients with low-grade serous cancer (7%), whereas 28% of patients with high-grade serous carcinoma presented with DTC ($P = 0.011$). The prevalence of DTC presence in other histologic subtypes is presented in Table 1.

BM Status and Progression-Free Survival

Patients were not considered for survival analysis if the follow-up period was less than 4 months. Thus, follow-up data were available in 456 cases. Only these patients were included

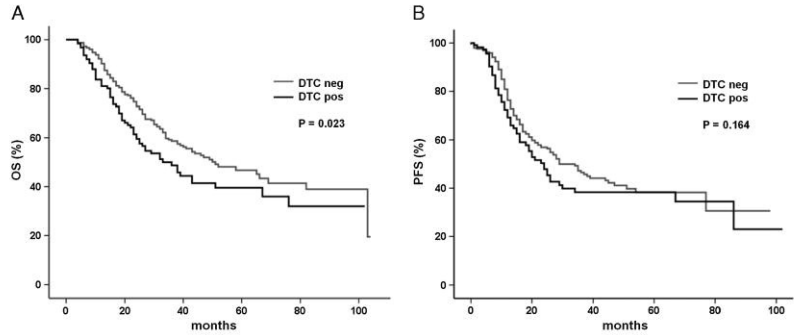

FIGURE 1. Kaplan-Meier survival analysis depending on BM status, P values calculated by the log-rank test (a, OS; b, PFS).

in the survival analysis. Median follow-up was 46 months (range, 4–104 months). Data on clinical outcome are summarized in Table 2. One hundred ninety-eight patients (43%) had recurrence during follow-up. Relapse was diagnosed in 48% of DTC-positive and 42% of DTC-negative patients ($P = 0.225$). Median progression-free survival (PFS) was shorter in patients with DTC in the BM (29 months; 95% CI, 22–36 months vs 24 months; 95% CI, 18–30 months; $P = 0.164$, determined by log-rank test; Fig. 1). In the multivariate regression analysis, BM status ($P = 0.030$), FIGO stage ($P < 0.001$), and resection status ($P = 0.003$) were the only independent predictors of PFS (Table 3).

BM Status and Overall Survival

One hundred ninety-six patients (43%) died during follow-up. Patients with DTC were more likely to die than BM-negative patients (51% vs 40%; $P = 0.029$). The median overall survival (OS) was significantly shorter among DTC-positive patients compared with DTC-negative patients (51 months; 95% CI, 37–65 months vs 33 months; 95% CI, 23–43 months; $P = 0.023$, determined by log-rank test). Survival curves are presented in Figure 1. Multivariate Cox regression model confirmed BM status as a strong independent predictor of shorter OS ($P = 0.001$). Age at diagnosis ($P = 0.004$), FIGO stage ($P < 0.001$), nodal status ($P = 0.022$), and resection status ($P < 0.001$), but not the grading, also predicted reduced OS in the multivariate analysis (Table 3). In the subgroup analysis, BM status was a strong predictor of OS in patients with high-grade serous carcinoma (295 patients; $P = 0.001$; Fig. 2). The subgroup analysis was not performed for other histologic types owing to small sample sizes. The prognostic effect of BM status was assessed within FIGO stages; for FIGO stage III to stage IV tumors (n = 299), a significant association with OS was confirmed ($P = 0.001$; Fig. 3). No significant correlation with OS was observed in FIGO I (n = 84) and FIGO II (n = 31) tumors when analyzed separately.

TABLE 3. Multivariate hazard ratios for death and relapse

Parameter	*P*	Hazard Ratio	95% CI for Hazard Ratio	
			Lower	Upper
PFS				
DTC status (positive vs negative)	0.030	1.490	1.038	2.137
FIGO stage (III–IV vs II vs I)	<0.001	2.966	1.992	4.416
Nodal status (positive vs negative)	0.386	0.855	0.600	1.218
Grading (G3 vs G2 vs G1)	0.844	1.030	0.765	1.388
Resection status (R2 vs R1 vs R0)	0.003	1.396	1.122	1.738
Age (70–89 vs 50–69 vs 30–49 vs 0–29	0.553	0.930	0.731	1.183
OS				
DTC status (positive vs negative)	0.001	1.892	1.321	2.712
FIGO stage (stage 3–4 vs 2 vs 1)	<0.001	4.055	2.103	7.819
Nodal status (positive vs negative)	0.022	1.585	1.068	2.354
Grading (G3 vs G2 vs G1)	0.765	1.049	0.767	1.434
Resection status (R2 vs R1 vs R0)	<0.001	1.741	1.401	2.163
Age (70–89 vs 50–69 vs 30–49 vs 0–29)	0.004	1.486	1.134	1.948

BM status and established prognostic factors, such as FIGO stage, nodal status, resection status, age at diagnosis, and grading were included in this statistic model. a) progression-free survival; b) overall survival.

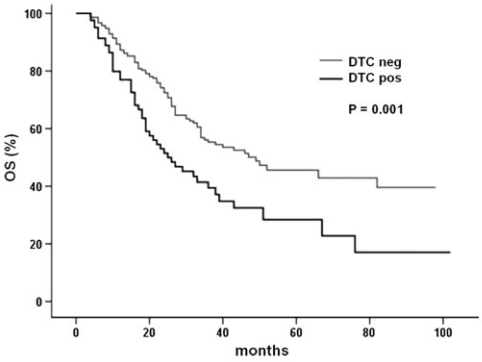

FIGURE 2. Subgroup analysis: high-grade serous carcinoma (n = 295); Kaplan-Meier OS analysis.

DISCUSSION

This is the largest pooled analysis so far on the prognostic relevance of DTC in primary ovarian cancer. A total of 495 patients were included in this prospective 3-center analysis. Disseminated tumor cells, as a surrogate parameter for minimal residual disease, could be detected in 27% of the patients, irrespective of the stage of the disease.

Other studies reported DTC incidence ranging from 20% to 60% in primary ovarian cancer, depending on the methodology and patients' collective.[4–6,8,13–16] Interestingly, ovarian malignancies rarely cause bone metastases.[10] This suggests that BM serves in these patients rather as a temporary "homing site" for isolated tumor cells, from where they are able to migrate and subsequently cause metastasis or locoregional recurrence. Because hematogenous dissemination of isolated tumor cells may already be observed in FIGO stage I, it may be hypothesized that contrary to the assumed natural history of ovarian cancer, single tumor cells acquire the potential to

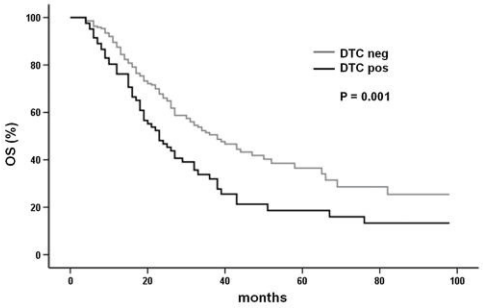

FIGURE 3. Subgroup analysis: patients with FIGO stages III to IV (n = 299); Kaplan-Meier OS analysis.

disseminate to extraperitoneal sites very early in the process of the disease.[5] Because DTCs may spread by means of the blood stream, we cannot exclude that those might also be able to repopulate the peritoneal cavity, an environment that easily supports ovarian cancer growth.

Influence of DTC Detection on Clinical Outcome

For ovarian cancer, data on the prognostic value of DTCs are so far limited (Table 4). In the present study, presence of DTCs predicted significantly shortened OS ($P = 0.001$ in the multivariate survival analysis). This is in accordance with several smaller studies. Braun et al[5] demonstrated impaired prognosis with regard to distant disease-free survival (DFS) in BM-positive patients at the time of diagnosis; in a subset of 64 optimally debulked patients, DTC presence remained a strong prognostic factor ($P = 0.002$), which highlights the role of DTC detection especially in patients who received successful surgical cytoreduction. We previously reported a significant correlation of positive BM status with reduced DFS in a group of 112 patients with FIGO stage I to stage III ovarian cancer.[4] Interestingly, in some studies, the presence of isolated tumor cells in secondary sites such as BM and blood was also associated with higher risk for local recurrence as well.[4,7] Therefore, it might be speculated that hematogenous tumor cell dissemination may serve as an indicator of a more aggressive phenotype of the primary disease that is likely to cause local relapse. In contrast, other authors reported no significant correlation between DTC detection and clinical outcome in ovarian cancer.[13,17] This discrepancy might be due to differences in study protocols, for example, time point of BM sample collection (preoperative vs postoperative aspiration). Hypothetically, a transient increase in cancer cell dissemination from the primary tumor due to intraoperative manipulation could contribute to false-positive results and therefore affect further analysis.[18]

Interestingly, in the current analysis, BM status did not influence PFS. This may be due to inconsistent relapse diagnosis. Most patients were treated within a clinical trial;

TABLE 4. Prognostic relevance of DTC and CTC in ovarian cancer

Author	No. Patients	Method	Median Follow-up (Months)	Positivity Rate (%)	Prognostic Significance
Our study	456	DTC (ICC)	46	27	OS, PFS*
Banys et al[4]	112	DTC (ICC)	12	25	DFS
Braun et al[5]	108	DTC (ICC)	45	30	DFS
Aktas et al[6]	95	DTC (ICC)	28	35	ns
Schindlbeck et al[8]	90	DTC (ICC)	28	23	DDFS
Marth et al[13]	73	DTC (immunobeads)	25	21	ns
Wimberger et al[14]	62	DTC (ICC)	18	54	DFS†
Poveda et al[7]	216	CTC (ICC: CellSearch)‡		14§	PFS, OS
Marth et al[13]	90	CTC (immunomagnetic beads)	25	12	ns
Aktas et al[6]	86	CTC (multiplex-RT-PCR: AdnaTest)	28	19	OS‖
Heubner et al[16]	68	Circulating 20S-proteasomes	19	—	OS
Fan et al[15]	66	CTC (immunofluorescence and cell invasion assay)	18	61	DFS
Wimberger et al[14]	62	Circulating nucleosomes, DNA, protease and caspase activity	18	—	DFS, OS

*Determined by multivariate Cox regression analysis.
†Disseminated tumor cells detected after chemotherapy.
‡Relapsed ovarian cancer.
§Two or more CTCs.
‖Both before and after chemotherapy.
DDFS, Distant disease-free survival; ICC, immunocytochemistry; ns, not significant; RT-PCR, reverse-transcriptase polymerase chain reaction

these patients received intensified follow-up with computed tomographic scans at regular intervals. In the group of patients treated outside clinical trials, follow-up care was carried out according to national guidelines and was based on clinical examination and patients' symptoms. Possibly, relapse was diagnosed later in the group treated outside clinical trials.

Disease Monitoring

Beyond the prognostic value of DTC detection, monitoring of minimal residual disease during and after treatment offers the opportunity to assess the residual risk of relapse. The presence of DTCs and/or CTCs may indicate persistent occult tumor load after treatment and thus an insufficient therapy response. Wimberger et al[12] assessed changes in DTC levels before and after first-line chemotherapy; marked increase in DTC numbers predicted significantly shorter PFS. Whether the reevaluation of the DTC status after completion of therapy may contribute to selection of patients who might benefit from extended or intensified treatment remains to be investigated.

Stem Cell Hypothesis

A provocative hypothesis has been introduced recently with respect to natural history and progression of ovarian cancer. While the "classical" stochastic model of cancer development holds that any cell may become a source of malignant transformation, emerging evidence supports the view that only a minor subpopulation of cancer cells has the potential to initiate cancer growth. These cells, called cancer stem cells (CSCs), have the ability to self-renew, propagate tumorigenesis, and are usually drug-resistant.[19] Experimental studies on stem cell biology have given new impetus to the cancer stem cell theory. Cancer stem cells are assumed to play an important role in the development of various tumor entities, such as breast and gastrointestinal cancer, retinoblastoma, and ovarian cancer.[20,21] Interestingly, ovarian cancer cell lines feature "side population" cells with potential to differentiate into cancers with different histologies, suggesting the pluripotent character of stem cells.[22]

One currently debated hypothesis is the theory that DTC/CTC, the surrogate marker for minimal residual disease and possibly precursor of systemic metastasis, represent in fact cancer stem cells. Most DTCs in breast cancer seem to express a putative CSC phenotype, such as ALDH1 positivity or presence of CD44 and absence of CD24, whereas CTCs were reported to exhibit stem cell and epithelial-mesenchymal transition markers.[23,24] Several characteristics of ovarian cancer (eg, high recurrence rates and multidrug resistance) suggest that the disease might be initiated and maintained by a unique population of cells with stem cell–like properties.[21] Advanced ovarian cancer generally responds to platinum-based combination therapy; however, this initial regression is often followed by emergence of therapy-resistant cell clones. One possible explanation for this phenomenon is the CSC-induced drug-resistance: standard therapies fail to target tumor-initiating cells.[21]

CONCLUSIONS

Despite advances in surgical and systemic therapy, ovarian cancer leads to relapse in 60% of patients within 5 years, resulting in poor OS. Currently, therapy efficacy is assessed by physical examinations, imaging, and evaluation of CA125 levels. New biomarkers are thus necessary for better prediction and prognostication.

Early hematogenous tumor cell dissemination is a common phenomenon observed in most solid tumors. Recent data support the clinical relevance of these cells; in the present report, we demonstrate significant impact of the presence of DTCs in the BM on survival. Whether these patients might benefit from extended or more aggressive therapy remains to be evaluated in future trials.

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3.3 Analysis of disseminated tumor cells before and after platinum based chemotherapy in primary ovarian cancer. Do stem cell like cells predict prognosis?

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Analysis of disseminated tumor cells before and after platinum based chemotherapy in primary ovarian cancer. Do stem cell like cells predict prognosis?

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ABSTRACT

Background: We recently reported that the presence of disseminated tumor cells (DTCs) in the bone marrow (BM) of primary ovarian cancer patients (POC pts) correlated with reduced progression free survival (PFS) and overall survival (OS). Here we analyzed whether the negative prognostic influence was related to DTC persistence after *platinum based chemotherapy* and/or due to DTCs associated with stem cell character.

Results: DTCs were detected in 33/79 pts (42%) before and in 32/79 pts (41%) AT. Persistent DTCs were found in 13 pts, 20 pts were only positive BT, 19 pts AT and 27 pts had no DTCs. Whereas the presence of DTCs BT significantly correlated with reduced OS ($p = 0.02$), pts initially DTC_{neg} BT but DTC_{pos} AT had a significantly shorter PFS ($p = 0.03$). DTC persistence resulted in a shorter PFS and OS reaching borderline significance ($p = 0.06$; $p = 0.07$). LIN-28-and SOX-2 positive cells were detected in all eight pts AT.

Patients and Methods: 79 POC pts were studied for DTCs before therapy (BT) and after therapy (AT) using immunocytochemistry. Eight pts harboring at least five DTCs AT were further analyzed on two additional slides by four-fold immunofluorescence staining for DAPI, Cytokeratin (CK), SOX-2 or LIN-28, CD45 and CD34 (Cy5). A stem-like tumor cell was classified as $Dapi_{pos,}$ $CD45_{neg,}$ $CD34_{neg,}$ $SOX\text{-}2_{pos}$/$LIN\text{-}28_{pos}$ and CK_{pos} or $CK_{neg.}$

Conclusions: Stem cell associated proteins are expressed in DTCs that are present AT and their presence seem to be correlated with a worse outcome. Additional therapeutic regimens may be necessary to eliminate these cells.

INTRODUCTION

Ovarian cancer is the fifth leading cause of all cancer related deaths in Europe and the United States and most tumors are diagnosed in an advanced stage with poor prognosis for the patients [1]. Conventional therapy is based on an initial debulking surgery aiming at macroscopic complete resection combined with subsequent platinum- and paclitaxel -based chemotherapy [2]. Postoperative residual tumor is one of the most important prognostic factors in advanced ovarian cancer [3, 4, 5].

It is hypothesized that cancer malignancy and metastasis are driven by a small subgroup of highly tumorigenic cells within the tumor, called metastasis initiating cells (MIC). These cells have the ability to self-renew, enhance tumorigenesis and are often found to be drug resistant [6]. The presence of such a small

population, often referred to as cancer stem cells (CSC), has been confirmed in ovarian cancer cell lines as well as in tumor tissue [7, 8]. Their amount is increased in chemotherapy resistant ovarian cancer cell lines [7] and they are believed to contribute to an aggressive behavior of epithelial ovarian cancer [9]. The pluripotency associated stem cell factors SOX2 (sry related) and LIN-28 have been found to be expressed in ovarian cancer cell lines and tissue [10, 11, 12]. Bareiss et al., showed that SOX2 expression is a CSC marker in serous ovarian carcinomas (SOC) and can induce CSC properties [11]. In addition, SOX2 was reported to enhance migration and invasion of ovarian cancer cells [13]. Importantly, SOX2 overexpression was shown to be a poor prognostic marker in ovarian cancer [14] and also shown to be involved in taxane resistance [15, 16].

In ovarian cancer, the primary tumor usually metastasizes to the peritoneum, but a variety of studies including ours indicate that tumor cells frequently disseminate into the bone marrow (BM). Disseminated tumor cells (DTCs) in the BM are detected in 20% to 60% of cases before the onset of platinum-based chemotherapy depending on the method of detection used. Their prognostic relevance with regard to reduced progression free survival (PFS) and overall survival (OS) has previously been demonstrated [17, 18, 19, 20, 21]. In addition, we demonstrated that patients with a marked increase of DTCs after platinum-based chemotherapy showed a significantly reduced PFS [22].

Based on the studies mentioned above, there is increasing evidence that DTCs could reflect cancer progression. Thus, DTCs could be used as novel targets for additional therapeutic strategies. In this study, we analyzed whether our previously reported negative prognostic influence of DTCs with regard to reduced PFS and OS 1) was related to the persistence of DTCs after platinum based chemotherapy and/or 2) might have arisen from a cellular phenotype showing stem cell characteristics.

RESULTS

Detection of DTCs

Before therapy (BT), DTCs were detected in 33/79 patients (42%) with a median number of 4 DTCs (range 1–37). After therapy (AT), 32/79 patients (41%) were positive for DTCs (median cell number of 8 cells (range 1–100) (Table 1). DTCs were found in 13 patients BT and AT, in 20 patients only BT and in 19 patients only AT, respectively. DTCs were not detected in samples taken BT or AT from 27 patients (Table 2).

Prognostic significance of DTCs

After a median follow up time of 62 months (range 10–128 months), 44 patients (56%) were still alive and 33 patients (42%) had died. The median follow-up time for PFS was 15 months (range 4–87 months) resulting in 53 (67%) relapses while 25 patients (32%) had no relapse (Table 1). The presence of DTCs BT significantly correlated with reduced OS ($p = 0.02$) and patients initially DTC_{neg} BT but DTC_{pos} AT had a significant shorter PFS ($p = 0.03$) (Table 2 and Figure1). The persistence of DTCs resulted in a shorter PFS and OS reaching borderline significance ($p = 0.06$; $p = 0.07$).

Evaluation of LIN28- and SOX-2-positive cells

Staining of patient samples is shown in Figures 2–5. Controls are shown in Supplementary Figures S1–S3. A DTC was classified as a stem-like tumor cell if it had the following staining characteristics: $Dapi_{pos}$, $CD45_{neg}$, $CD34_{neg}$, $SOX\text{-}2_{pos}$/$LIN\text{-}28_{pos}$ and CK_{pos} or CK_{neg} (Figures 2–5). The Kasumi cell line was used to establish CD34 expression (Supplementary Figure 1) and BM samples from healthy donor patients for CD34- and CD45-expression (Supplementary Figures 2 and 3).

Detection of LIN-28- and SOX-2-positive cells

DTCs from 10 patients were analyzed BT and AT for SOX-2 and LIN-28 positive cells AT (Table 3; columns 1 and 2; Supplementary Table 1). 8/10 patients had at least five DTCs as detected by immunocytochemistry using A45/B-B3. In addition, 2/10 patients (patient 2 and 5) were DTC_{neg} AT but DTC_{pos} BT. As apparent from Table 3, AT CK_{pos}/$LIN\text{-}28_{pos}$ cells were detected in 9/10 patients [median 2 cells (range 1–5)] and CK_{neg}/$LIN\text{-}28_{pos}$ cells in 7/10 patients [median 3 cells (range 1–11)], respectively. CK_{pos}/$SOX\text{-}2_{pos}$ cells were detected in 6/8 patients [median 2 cells (range-0–4)] and CK_{neg}/$SOX\text{-}2_{pos}$ cells were found in 7/8 patients [median 4 cells (range 1–11)]. Patients two and five, who were characterized as DTC_{neg} AT by immunocytochemistry but were positive BT (37 and 6 DTCs, respectively) were included in our analysis for stem cell- associated markers. Interestingly, these two patients harbored 1–2 $LIN\text{-}28_{pos}$ and $SOX\text{-}2_{pos}$ cells in their BM AT. Thus, we evaluated LIN-28-/SOX-2-positive cells BT in cases vice versa, DTC_{neg} BT but DTC_{pos} AT (patients 1, 3 and 4) as well as in patient number 6 with persistent DTCs. As shown in Table 3, in patients who switched initially DTC_{neg} before but becoming DTC_{pos} AT (patients 1, 3 and 4), in patients who switched from being DTC_{pos} before but becoming DTC_{neg} AT (patients 2 and 5) as well as in patient number 6 with persistent DTCs (patient 6) a few LIN-28 as well as SOX-2-positive cells were present in BM even BT.

DISCUSSION

To the best of our knowledge, this is the first study showing that DTCs, present after platinum based chemotherapy in primary ovarian cancer patients show

Table 1: Patient characteristics at the time of primary diagnosis

Total	79
Age	median 60 years, (26–86)
FIGO stage	
I–II	21 (26%)
III	48 (61%)
IV	10 (13%)
Nodal status	
N_o	32 (40,5%)
N_1	32 (40,5%)
N_x	10 (19%)
Grading	
I–II	44 (56%)
III	33 (42%)
Unknown	2 (2%)
Residual tumor	
Macroscopic	
Complete resection	49 (62%)
Any residual tumor	30 (38%)
Histologic type	
Serous	47 (60%)
Mucinous	9 (11%)
Other	23 (29%)
DTC	
Before therapy	33 (42%)
After therapy	32 (41%)
Survival	
PFS[1]	median 15 months, (4–87 months)
OS[2]	median 62 months, (10–128 months)
Alive	44 (56%)
Dead	33 (42%)
Unknown	2 (2%)
Recurrence	
No relapse	25 (32%)
Relapse	53 (67%)
Unknown	1 (1%)
Platinum resistance	
Platinum sensitive	60 (76%)
Platinum resistant	19 (24%)

[1]PFS: progression-free survival, [2]OS: overall survival.

Table 2: Prognostic significance of DTCs before and after therapy with regard to PFS and OS

Status	Number of patients (*n*)	PFS (*p*-value)	OS (*p*-value)
Total	79		
DTC_{pos} before therapy	33	0.06	**0.02**
DTC_{pos} after therapy	32	0.35	0.98
DTC_{pos}/DTC_{pos}	13	0.06	0.07
DTC_{neg}/DTC_{neg}	27	0.77	0.31
DTC_{pos}/DTC_{neg}	20	0.46	0.25
DTC_{neg}/DTC_{pos}	19	**0.03**	0.18

stem cell characteristics. Furthermore, although *p* values reached borderline significance, these cells might be associated with worse outcome which finally has to be proven in a bigger patient cohort.

The rate of DTC detection in primary ovarian cancer before the administration of platinum-based chemotherapy has been reported to be 20% to 60%, depending on the method of detection used. Furthermore, their presence has been associated with worse outcome [17, 18, 19, 20, 21, 22]. The lack of significant correlation between DTCs and clinical outcome reported by other investigators may be due to their use of different antibodies for detection of DTCs [23, 24].

In this study, DTCs were present in the BM AT in 41% of the patients which is in accordance with our earlier data which also demonstrated that DTCs, still present AT, were non-apoptotic and their marked increase was associated with a significantly reduced PFS [22]. These findings suggest that the BM seems may to be a temporary homing site for isolated tumor cells, where they can persist and potentially induce recurrence of the disease. Analyzing 79 patients BT and AT, we confirm the negative prognostic influence of DTC detection with regard to OS [20]. We observed persistent DTCs in 16% of the patients which was associated with a shorter PFS and OS, reaching borderline significance. Interestingly, 24% of the patients that were initially characterized as DTC_{neg} BT and converted to DTC_{pos} AT had a significantly shorter PFS. The negative prognostic influence of these cells could be in alliance with a currently discussed hypothesis that some DTCs may have cancer stem cell features and may be the active source of metastatic spread in primary tumors, in addition to resistance to various chemotherapeutic agents and radiotherapy [25]. Two studies have confirmed a putative stem cell phenotype among DTCs in breast cancer patients [26, 27]. Here, we demonstrate that presence of DTCs that persist AT express the stem cell markers Lin-28 and/or SOX-2. We show that stem cell-like cells were present before the administration of chemotherapy. These findings may explain the significantly shorter PFS of patients who changed from DTC_{neg} BT to DTC_{pos} AT. In addition, patients characterized as DTC_{neg} AT also harbored some Lin-28 and/or SOX-2 positive cells in their BM which may be responsible for a worse outcome. Until now, tumor stem cells have only been analyzed in ovarian tumor tissue, but not in DTCs. In this regard, previous studies have shown that LIN-28, SOX-2 as well as OCT-4 play a major role in carcinogenesis [10, 11, 12]. Wang et al., reported that SOX-2 targets SRC Kinase, a non-receptor tyrosine kinase that increases cell migration, invasion and adhesion of serous ovarian carcinoma cells [13]. Inhibition of either LIN-28 or Oct-4 expression decreases cell viability. The combined repression of both LIN-28 and Oct-4 results in synergistic inhibition of cancer cell growth and survival of ovarian cancer cell lines [9]. Expression of SOX-2 has been investigated by immunohistochemistry analysis of normal ovarian epithelial, serous and mucinous cystadenoma and cystadenomacarcinoma specimens [28] and LIN-28 was overexpressed in different epithelial tumors including breast, lung, colon and ovarian cancer [29]. Furthermore, SOX-2 may be crucial for the development of chemotherapy resistance. Yang et al., analyzed SOX-2 expression in clinical tissue samples and ovarian cancer cell lines using immunohistochemistry and real-time PCR and demonstrated that SOX-2 was overexpressed in paclitaxel-resistant cells [30]. In ovarian cancer patients receiving taxanes, expression of SOX-2 was shown to be correlated with chemotherapy resistance and a shorter PFS whereas patients receiving non-taxane based chemotherapy showed no significant response influence [31]. Since the patients in our study have also received a combined therapy with paclitaxel and carboplatin, it may be possible that SOX-2 expression on DTCs was associated with chemotherapy resistance. However, mechanisms associated with chemotherapy resistance in ovarian cancer still remain unclear. Functionally, primary platinum-resistance, defined as platinum-free treatment interval of less than 6 months observed in up to 20% of ovarian cancer patients, can be the result of either increased tolerance towards DNA-platinum-adducts or enhanced DNA-repair capacity of tumor cells [32, 33, 34]. In this context, we recently demonstrated that $ERCC1_{pos}$ (excision-repair cross-complementing rodent repair deficiency, complementation group 1 nuclease) circulating

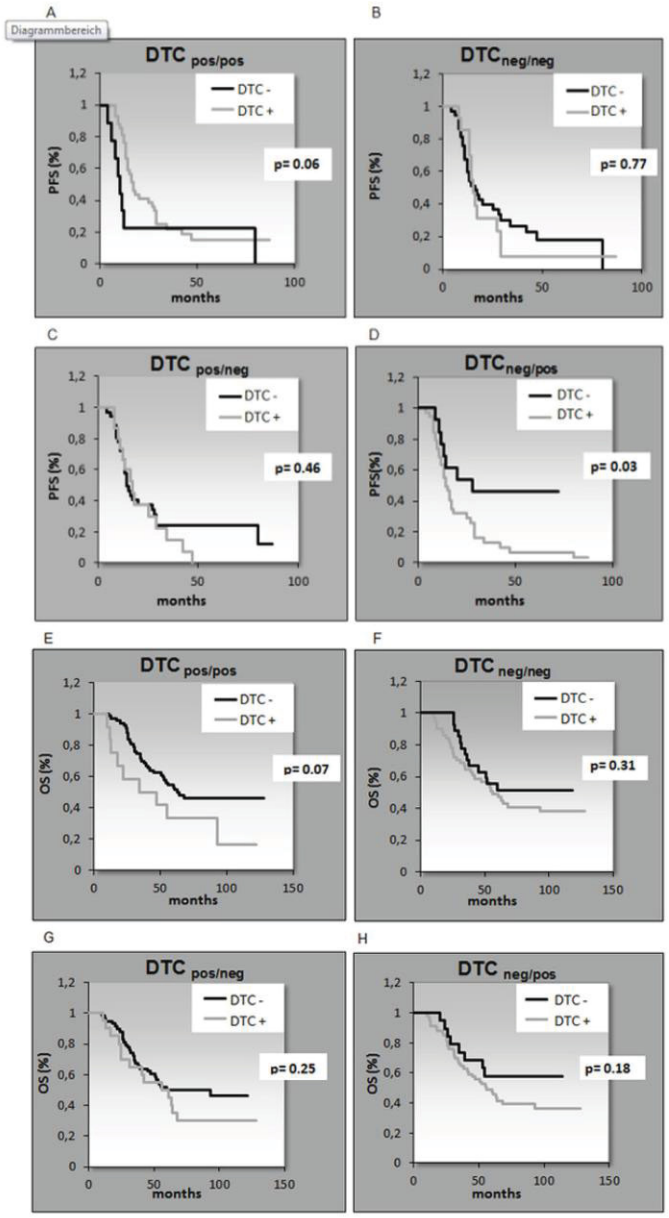

Figure 1: Kaplan-Meier analysis for the correlation of PFS (A–D) and OS (E–H) with DTC detection. Patients initially DTC_{neg} before therapy but DTC_{pos} after therapy had a significant shorter PFS ($p = 0.03$) (Figure 1D). A. PFS $DTC_{pos/pos,}$ B. PFS $DT_{neg/neg,}$ C. PFS $DTC_{pos/neg,}$ D. PFS $DTC_{neg/pos,}$ E. OS $DTC_{pos/pos,}$ F. OS $DTC_{neg/neg,}$ G. OS $DTC_{pos/neg,}$ H. OS $DTC_{neg/pos}$.

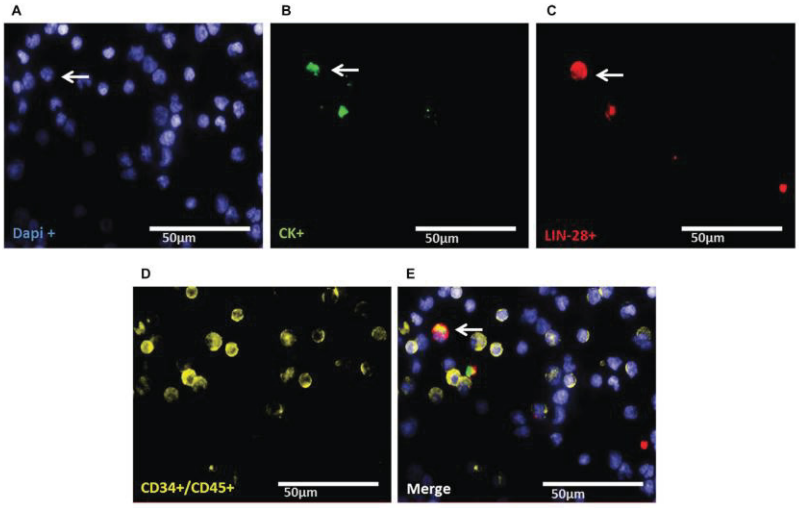

Figure 2: Representative four-fold immunofluorescence staining for CK_{pos}/LIN-28_{pos} cells after therapy of patient No1. (**A**) Cell nuclei were stained with Dapi. (**B**) Indicates a CK_{pos} cell. (**C**) Alludes a cell with LIN-28_{pos} phenotype. (**D**) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. (**E**) Indicates a merge of a DTC with the phenotype $Dapi_{pos}$, CK_{pos}, LIN-28_{pos}, $CD34_{neg}$ and $CD45_{neg}$, magnification at 63×.

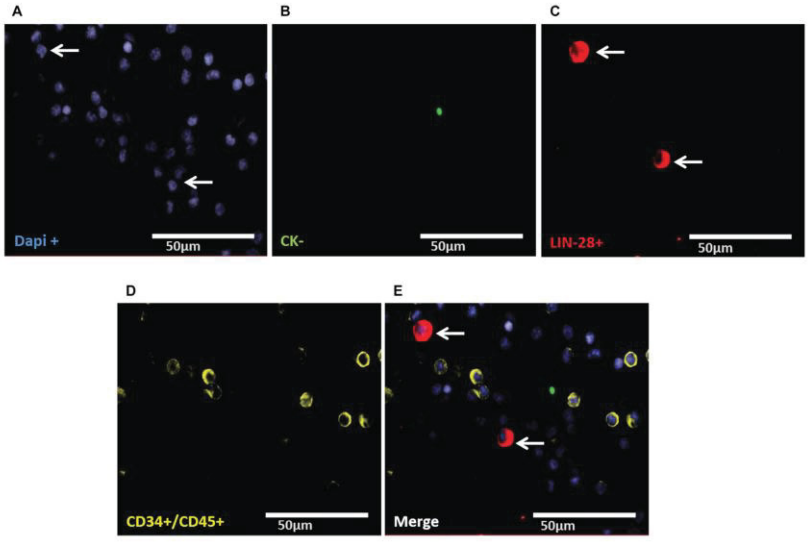

Figure 3: Representative four-fold immunofluorescence staining for CK_{neg}/LIN-28_{pos} cells after therapy of patient No1. (**A**) Cell nuclei were stained with Dapi. (**B**) Indicates a CK_{neg} cell. (**C**) Alludes a cell with LIN-28_{pos} phenotype. (**D**) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. (**E**) Indicates a merge of two DTCs with the phenotype $Dapi_{pos}$, CK_{neg}, LIN-28_{pos}, $CD34_{neg}$ and/or $CD45_{neg}$, magnification at 63×.

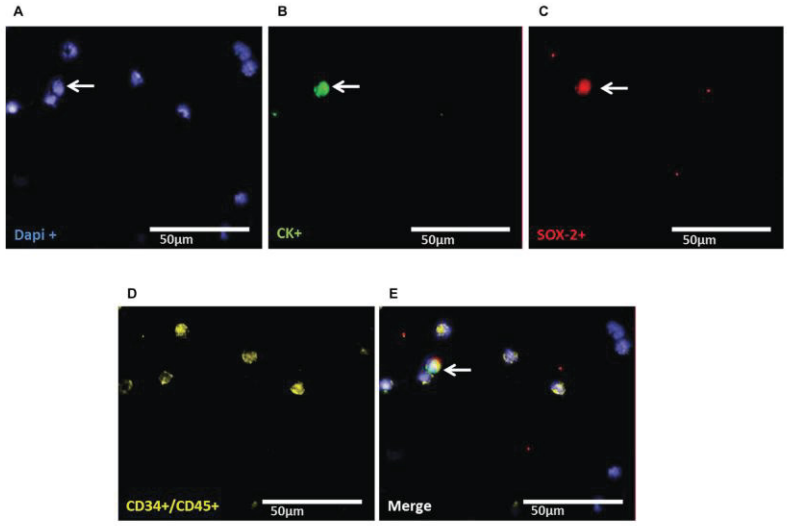

Figure 4: Representative four-fold immunofluorescence staining for CK_{pos}/SOX-2_{pos} cells after therapy of patient No1. (**A**) Cell nuclei were stained with Dapi. (**B**) Indicates a CK_{pos} cell. (**C**) Alludes a cell with SOX-2_{pos} phenotype. (**D**) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. (**E**) Indicates a merge of a DTC with the phenotype $Dapi_{pos}$, CK_{pos}, SOX-2_{pos}, $CD34_{neg}$ and/or $CD45_{neg}$ magnification at 63×.

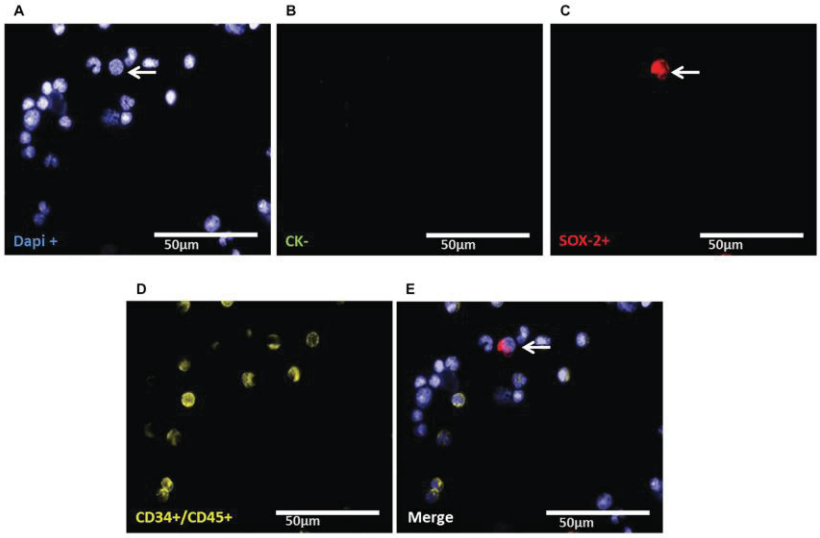

Figure 5: Representative four-fold immunofluorescence staining for CK_{neg}/SOX-2_{pos} cells after therapy of patient No1. (**A**) Cell nuclei were stained with Dapi. (**B**) Indicates a CK_{neg} cell. (**C**) Alludes a cell with SOX-2_{pos} phenotype. (**D**) Shows $CD34_{pos}$ and/or $CD45_{pos}$ cells. (**E**) Indicates a merge of a DTC with the phenotype $Dapi_{pos}$, CK_{neg}, SOX-2_{pos}, $CD34_{neg}$ and $CD45_{neg}$ magnification at 63×.

Table 3: Distribution of DTCs and LIN-28-/SOX-2-positive cells before and after therapy

			before therapy				after therapy			
Patient	DTC_{pos} before therapy (A45-B/B3)	DTC_{pos} after therapy (AF45-B/B3)	CK_{pos}/LIN-28_{pos}	CK_{neg}/LIN-28_{pos}	CK_{pos}/SOX-2_{pos}	CK_{neg}/SOX-2_{pos}	CK_{pos}/LIN-28_{pos}	CK_{neg}/LIN-28_{pos}	CK_{pos}/SOX-2_{pos}	CK_{neg}/SOX-2_{pos}
1	0	14	25	5	24	2	4	0	3	4
2	37	0	2	0	4	1	0	1	2	0
3	0	11	8	2	5	1	2	0	0	1
4	0	15	2	1	1	1	2	1	nsa	nsa
5	6	0	1	1	2	1	1	0	2	1
6	28	18	nsa	nsa	2	3	3	7	4	11
7	0	5	nsa	nsa	nsa	nsa	1	3	0	2
8	0	100	nsa	nsa	nsa	nsa	5	9	1	2
9	1	35	nsa	nsa	nsa	nsa	2	1	nsa	nsa
10	0	10	nsa	nsa	nsa	nsa	3	11	2	9

nsa: no slides available.

tumor cells (CTCs) constituted an independent predictor, not only for OS but also for PFS in our ovarian cancer patients. Most interestingly, the presence of $ERCC1_{pos}$ CTCs at primary diagnosis was an independent predictor for platinum-resistance whereas ERCC1-expression in the corresponding primary tumor tissue predicted neither platinum-resistance, nor prognosis [35]. Consequently, assuming that CTCs must be spread into the circulation from existing pools in secondary organs, e.g. the BM, one might speculate that $ERCC1_{pos}$ DTCs also exist and contribute to platinum resistance. Interestingly, DTCs present BT in our patients significantly correlated with clinical platinum resistance (data not shown).

In this study, we also detected CK_{pos}/SOX-2_{pos} (LIN-28_{pos}) as well as CK_{neg}/SOX-2_{pos} (LIN-28_{pos}) cells in all patients. We assume that two different cell types with expression of stem cell associated proteins may have been detected. It has been described that tumor cells undergo phenotypic changes, known as epithelial-mesenchymal transition (EMT), which allow them to migrate to sites of metastasis without being eliminated by conventional treatment [36]. Thus, CK_{neg}/SOX-2_{pos} (Lin-28_{pos}) cells might result from EMT while the CK_{pos}/SOX-2_{pos} (LIN-28_{pos}) epithelial phenotype may have remained unchanged.

Taking all these considerations into account, additional therapeutic strategies will be required to target signaling pathways concerning CSC. These studies will include mTOR inhibitors, acting downstream of the PI3K/AKT pathway [37], salinomycin [38] or a new synthetic curcumin analogue against ALDH1 and GSK-3ß [39]. Finally, approaching the tumor microenvironment, such as interrupting the immune cells and cytokines (e.g. IL-6, IL-8) as well as the immune checkpoints (PD1/PDL1) may provide additional new tools for immunological killing of cancer stem cells [40, 41, 42]

Conclusion and limitation of the study

The cohort of our patients is probably too small to draw the final conclusion that a significant selection of stem cell marker-positive DTCs occurs during chemotherapy. Consequently, the results presented here should be viewed as a "proof of principle", that DTCS with stem cell characteristics exist among DTCs that are present BT and persist AT. To the best of our knowledge, we are the only group that has a collection of BM cells harvested from primary ovarian cancer patients AT. In this regard, 79 paired samples from patients who consented to allow collection of their BM under local anesthesia AT for research purposes is a unique collection and would be difficult to achieve high patient numbers. Furthermore, based on the number of residual slides and methodological requirements, we only analyzed two stem cell markers. Ongoing studies will include other stem cell markers, such as OCT4 as well as resistance marker to finally elucidate the prognostic relevance of these cells.

PATIENTS AND METHODS

Patient characteristics

79 patients with primary ovarian cancer who presented at the Department of Gynecology and Obstetrics, University Hospital Essen between February 2004 and January 2010 were included in this analysis. Patient characteristics are documented in Table 1. The mean age was 60 years (range 26–86 years), the median follow-up time was 62 months (10–128 months) for OS and 15 months (4–87 months) for PFS. Written Informed consent was obtained from all patients and the study was approved by the Local Ethics Committee (05–2870). Tumors were classified according to the

WHO classification of tumors of the female genital tract. Grading was conducted using the grading system proposed by Silverberg [43] and tumor staging was classified according to the Fédération Internationale de Gynécology et d'Obstétrique (FIGO 2009). The entire study population underwent primary radical surgery. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy, peritoneal stripping was performed and in addition to pelvic and para-aortic lymphadenectomy, if macroscopic complete resection was achieved. The most important aim of surgery was to achieve macroscopic complete tumor resection. Radical pelvic and para-aortic lymphadenectomy were only performed if complete tumor resection was achieved intraperitoneally following actual guidelines (www.ago-ovar.de). All patients received at least six cycles of carboplatinum AUC 5 and paclitaxel 175 mg/m2. Tumors were clinically defined as platinum-resistant if they recurred within six months after the completion of platinum-based chemotherapy.

Cell lines

The human ovarian cancer cell line OVCAR-3 and the Kasumi-1 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 containing 10% (20% for Kasumi-1) fetal calf serum and 1% (100 U/ml) Penicillin-Streptomycin (Gibco™ by Thermo Fisher Scientific, Waltham MA, US) . Cells were grown at 37°C in a humidified atmosphere with 5% CO2.

Detection of DTCs

Between 10 and 20 ml BM were aspirated from the anterior iliac crests and processed within 24 hours. DTC selection and detection was performed based on the recommendations for standardized tumor cell detection [44]. Details of the staining procedure and cell detection have been described elsewhere [22]. Briefly, BM cells were isolated from heparinized BM (5000 U/ml BM) by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400 × g for 30 min. Slides were analyzed for DTCs by immunocytochemistry using the pan-cytokeratin antibody A45-B/B3. Microscopic evaluation of the slides (1×10^6 mononuclear cells per slide) was carried out using the ARIOL system (Applied Imaging) according to the ISHAGE evaluation criteria [45].

Detection of LIN-28- and SOX-2-positive cells

LIN-28- and SOX-2 positive cells were analyzed separately on additional slides of the same patients harboring at least five DTCs as detected by immunocytochemistry using the A45B-B3. Four-fold immunofluorescence staining was established using the OVCAR-3 cell line spiked into blood of healthy donors. Since CD34-positive normal hematopoietic stem cells comprise 1.5% of marrow mononuclear cells [46], we included CD34 in our analysis to exclude false-positive results. CD34 was analysed using the Kasumi-1 cell line since BM of healthy donors was difficult to obtain and only available from one donor (Supplementary Figure 1).

Slides were fixed with 4% Paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X-100 for 15 min and subsequently washed with TBS and Triton-X-100 three times for five min. Slides were stained with SOX-2 (Anti-human SOX-2, 1:50, R & D Systems, USA), LIN-28 (LIN-28, 1:350, Rabbit polyclonal ab46020, Abcam, UK), C11 (anti-PAN-Cytokeratin, 1:400, FITC-labelled, GeneTex, USA), CD34 (Alexa Flour anti-human 647, 1:100, Cy5 labelled, Biolegend, USA) and CD45 (Alexa Fluor 647;sc1178 Santa Cruz, USA) incubated in a wet chamber for one hour at room temperature. Subsequently, slides were incubated with DAPI [pre diluted in Phosphate Buffered Saline (1:250) and further diluted in AB diluent (1:20, Dako, Germany)], and TRITC-labelled donkey anti-goat 594 (SOX-2), donkey anti-rabbit 594 (LIN-28) (both1:100, Invitrogen, USA) under the same conditions followed by three washing steps for five minutes. Moreover, negative controls for primary antibodies were prepared by staining the spiked OVCAR-3 cell line with the secondary antibodies for 30 minutes under the same conditions. The slides were mounted with Dako fluorescent mounting medium s3023 and a coverslip and dried overnight in a cooling chamber. Counting was performed visually, using an immunofluorescence microscope (Axioplan 2 Imaging Zeiss Germany, Metasystems) and Isis Fish imaging system V5.3 (Meta Systems, Germany) at a magnification of 40× or 63×.

Statistical analysis

Survival analysis was performed by using Winstat (2012.1) an upgrade of Microsoft Excel. Survival intervals were screened from the time of BM aspiration at first diagnosis to the time of death or first time of relapse, defined as either local recurrence or distant metastasis. Kaplan-Meier curves were established using the log-rank test to evaluate univariate significance of the parameters.

CONFLICTS OF INTEREST

None.

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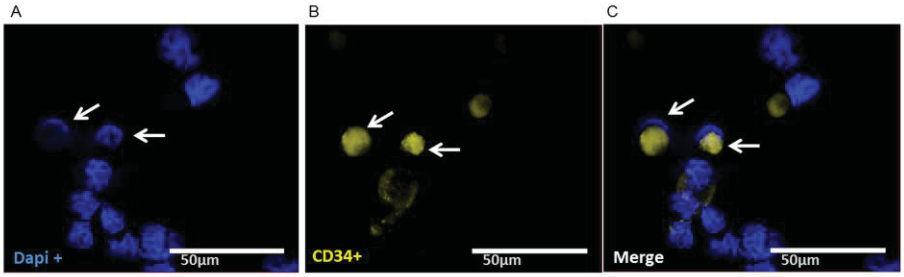
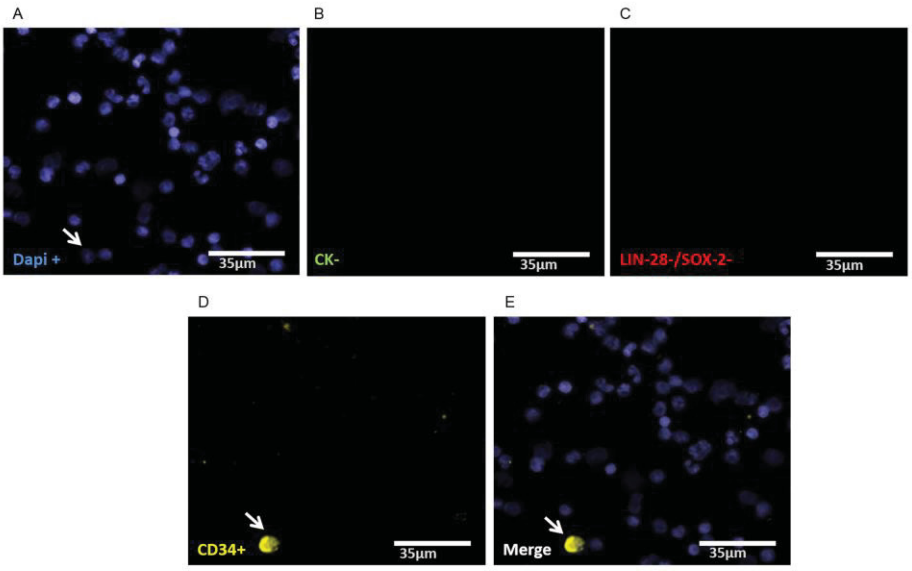
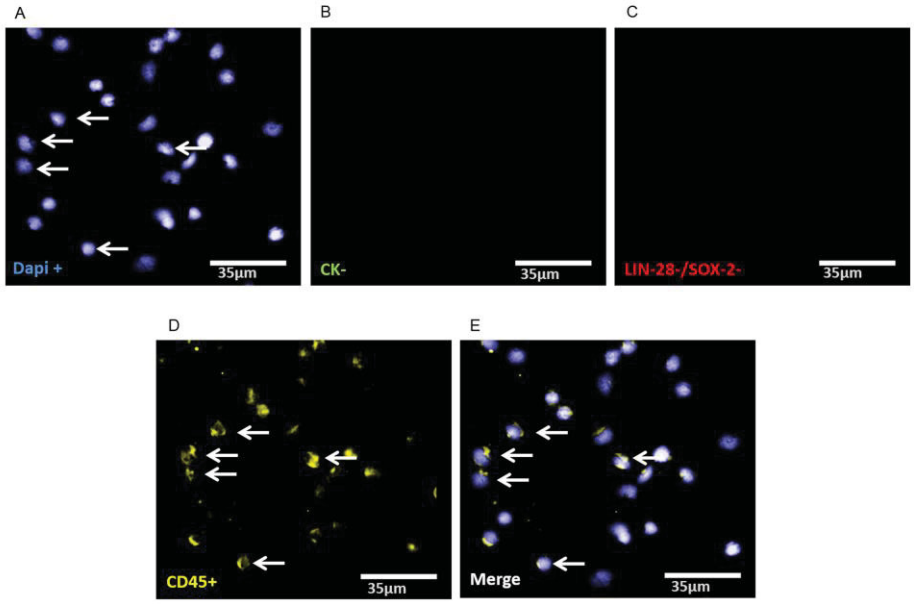
Supplementary Materials



Supplementary Figure S1: Representative staining of immunofluorescence staining of the kasumi-1 cell line used as positive control for the detection of hematopoietic stem cells ($CD34_{pos}$). (**A**) Cell nuclei were stained with Dapi. (**B**) The arrows show two $CD34_{pos}$ cells. (**C**) Indicates a merge of $Dapi_{pos}$ and $CD34_{pos}$ cells, magnification at 63×.



Supplementary Figure S2: Representative four-fold immunofluorescence staining for detection of hematopoietic stem cells ($CD34_{pos}$) in the bone marrow of a healthy donor using primary and secondary antibody for LIN-28 and SOX-2. (**A**) Cell nuclei were stained with Dapi. (**B**) Indicates CK_{neg} cells. (**C**) Alludes a cell with LIN-28_{neg}/SOX-2_{neg} phenotype. (**D**) Shows $CD34_{pos}$ cell. (**E**) Indicates a merge of a hematopoietic stem cell with $Dapi_{pos}$, CK_{neg}, SOX-2_{neg}/LIN-28_{neg}, $CD34_{pos}$ phenotype, magnification at 40×.



Supplementary Figure S3: Representative four-fold immunofluorescence staining for detection of leukocytes ($CD45_{pos}$) in the bone marrow of a healthy donor using primary and secondary antibody for LIN-28 and SOX-2. (**A**) Cell nuclei were stained with Dapi. (**B**) Indicates CK_{neg} cells. (**C**) Alludes a cell with $LIN\text{-}28_{neg}$/$SOX\text{-}2_{neg}$ phenotype. (**D**) Shows a $CD34_{pos}$ cell. (**E**) Indicates a merge of hematopoietic stem cells with $Dapi_{pos}$, CK_{neg}, $SOX\text{-}2_{neg}$/$LIN\text{-}28_{neg}$, $CD45_{pos}$ phenotype, magnification at 40×.

Supplementary Table S1: Characteristics of patients analyzed for DTCs with stem cell character

Total	10
Age	median 58 years, (30–77)
FIGO stage	
I–II	1 (10%)
III	6 (60%)
IV	3 (30%)
Nodal status	
N_0	3 (30%)
N_1	2 (20%)
N_x	5 (50%)
Grading	
I–II	7 (70%)
III	3 (30%)
Residual tumor	
Macroscopic	
Complete resection	4 (40%)
Any residual tumor	6 (60%)
Histologic type	
Serous	8 (80%)
Other	2 (20%)
Survival	
PFS[1]	median 18 months, (4–72 months)
OS[2]	median 53 months, (13–101 months)
Alive	4 (40%)
Dead	6 (60%)
Recurrence	
No relapse	2 (20%)
Relapse	8 (80%)

[1]PFS: progression-free survival, [2]OS: overall survival.

4. Findings and future directions

The presence of CTCs and DTCs in patients with ovarian cancer is documented by several publications, which in some studies could be correlated with poor clinical outcome. However, analysis did not go beyond simple cell detection/enumeration. Since CTC/DTCs with epithelial-mesenchymal-transition (EMT) and/or stem-like traits are thought to be involved in metastatic progression and recurrence, investigating their molecular profiles might help to identify therapy resistant CTCs/DTCs and to overcome treatment failure. Therefore, the aim of this work was to establish different procedures to detect ovarian cancer CTCs and DTCs and to characterize their phenotype.

Gene expression profiling of single circulating tumor cells in ovarian cancer - Establishment of a multi-marker gene panel

Findings

So far, the analysis of enriched bulk CTC populations is the most commonly used approach to detect and characterize CTCs (e.g. AdnaTest OvarianCancer/ AdnaTest EMT-1/StemCell), which was also used in this work. However, it turned out that the enriched CTC population was always contaminated with other blood-derived cells (e.g. leukocytes) resulting in to false observations and interpretations. For instance, stem cell- and EMT associated transcript signals were detected in blood samples of healthy donors. Due to their mesenchymal nature, leukocytes interfered with the detection of EMT transcripts and incompletely differentiated leukocytes contributed the detection of stem cell transcripts. Besides, it is important to note that, similar to the cellular heterogeneity of the primary tumor, CTCs themselves are likely to consist of a heterogeneous cell population.

To overcome these limitations, a workflow to isolate and profile gene expression of single CTCs from ovarian cancer patients was developed and established which allows discrimination of cells with different expression profiles and prevents from false observations. This workflow comprises of a density gradient-based enrichment for nucleated cells, a depletion of CD45-positive cells of hematopoietic origin and immunofluorescent labeling of CTCs by EpCAM and Muc-1. Single CTCs were then isolated by micromanipulation and processed by a multimarker gene panel to characterize the expression of different epithelial (EpCAM, Muc-1, CK5/7), EMT (N-cadherin, Vimentin, Snai1/2, CD117, CD146, CD49f) and stem cell (CD44, ALDH1A1, Nanog, SOX2, Notch1/4, Oct4, Lin28) associated transcripts.

The analysis of 77 single OvCar3 cells revealed heterogeneous gene expression patterns for several epithelial, stem cell- and EMT-associated transcripts. Moreover, co-expression of epithelial, mesenchymal and stem cell-associated transcripts was found in some cells. Fifteen single CTCs derived from three ovarian cancer patients were additionally characterized: they were positive for stem cell (CD44, ALDH1A1, Nanog, Oct4) and EMT-associated transcripts (N-cadherin, Vimentin, Snai2, CD117, CD146). Particularly, inter-cellular and inter-patient heterogeneity as well as co-expression of epithelial, mesenchymal and stem cell-associated transcripts in the same CTC was observed. Our results are in line with previous publications, which have already proposed heterogeneous CTC populations in other tumor entities, regarding morphology, molecular characteristics and metastatic potential. Moreover, these results support the hypothesis that different CTC subpopulations can be selected during *platinum*-based therapy and may be responsible for drug resistance.

Limitations and future investigations

The enrichment strategy for ovarian cancer CTCs presented in this work exclusively focuses on the epithelial-associated markers EpCAM and CK. Since in this and other work co-expression of mesenchymal and epithelial markers in CTCs after enrichment based on epithelial antigens was documented, more sophisticated enrichment strategies which enable the characterization of *all* CTCs, not only the subset of epithelial enriched CTCs, are needed. The enrichment strategy used herein is exemplarily, but should be combined with other enrichment approaches. For instance, a detection and isolation strategy for EpCAM negative breast cancer CTCs was recently established by our group and it is planned to adapt this method for ovarian cancer CTCs.

Several techniques for quantitative transcriptomic profiling of CTCs are available. However, they all require pre-amplification steps to increase the amount of mRNA for quantification and subsequent multiple transcript analysis. These amplifications are expensive and prone to technical errors due to an amplification bias. In contrast, the multiplex-RT-PCR approach presented in this work was designed as a “non-quantitative” assay with the intention to provide a time- and cost-efficient screening tool for molecular characterization of CTCs. It allows a simultaneous detection of 19 transcripts *without* prior pre-amplification. After screening of multiple patient-derived CTCs during therapy, therapy-relevant transcripts can be potentially identified and quantificated by RT-qPCR using the same primers.

Pooled analysis of the prognostic relevance of disseminated tumor cells in the bone marrow of patients with ovarian cancer

Tumor cell dissemination into the BM is a common phenomenon in ovarian cancer. Various clinical studies have provided evidence that the presence of DTCs detected at the time of tumor resection is associated with poor clinical outcome. In contrast, other research articles reported no significant correlation between DTC detection and clinical outcome. Hence, the clinical relevance of DTC analyses as a prognostic tool is still under debate.

Findings

The impact of the BM status on the survival of patients with ovarian cancer was evaluated within a prospective 3-center study. In total, 495 patients with primary ovarian cancer were included in the study. BM aspirates were obtained intraoperatively from the iliac crest and DTCs were identified by immunocytochemistry (nuclear, CK positive cells) and cytomorphological observations. DTCs were detected in 27% of all BM aspirates, ranging from 1 to 42 DTCs per 2x 10^6 mononuclear cells. Next, the clinical outcome was compared with the presence of DTCs in the BM and the analysis revealed that OS was significantly decreased in DTC-positive patients compared to DTC-negative patients (33 months vs. 51 months). Patients harboring DTC(s) were more likely to die than BM-negative patients (51%vs 40%; $p = 0.029$). Interestingly, the DTC status correlated with the histological subtype but with no other established clinicopathological factors. Besides, the subgroup analysis of high-grade serous carcinoma revealed that a positive BM status is a strong predictor for a decreased OS ($p = 0.001$).

Limitations and future investigations

This study clearly demonstrated that the presence of DTCs is associated with poor clinical outcome, with regard to OS. Nevertheless, the exact identification of ‘THE’ tumor cell with metastasis initiating capacity and the ability to induce recurrence remains challenging. Since ovarian cancer disposes a high recurrence rate and multidrug resistance, cancer stem cells most likely initiate and retain the disease. Therefore, their role during cancer progression needs to be further investigated in future studies. Besides their possible prognostic value, DTC detection could be also useful to monitor minimal residual disease during and after treatment and to estimate the residual risk of relapse. Especially, the presence of DTCs after therapy could be used as an indicator for an insufficient therapy response.

Analysis of disseminated tumor cells before and after platinum based chemotherapy in primary ovarian cancer. Do stem cell like cells predict prognosis?

Findings

This study further analyzed whether the negative prognostic influence of DTCs on the PFS and/or OS is related to their persistence after *platinum*-based chemotherapy and/or associated with stem cell character. Therefore, the presence of DTCs before (BT) and after therapy (AT) was evaluated on 79 primary ovarian cancer patients by immunocytochemistry. DTCs were detected in 42 % BT and in 41% AT. The persistence of DTCs was found in 17% of all patients, 25% were only positive BT, 24% AT and 34% harbored no DTCs. The presence of DTCs BT was significantly correlated with reduced OS ($p = 0.02$). Patients who were DTC-negative BT but DTC-positive AT showed a significant shorter PFS ($p = 0.03$), while DTC persistence revealed a shorter PFS and OS with borderline significance ($p = 0.06$; $p = 0.07$).

In order to investigate a possible stem cell character of DTCs, eight patients with at least five DTCs and 2 patients with no DTCs AT were additionally analyzed by four-fold immunofluorescence staining for DAPI, CK, SOX2 or LIN-28, CD45 and CD34 (Cy5). A stem-like tumor cell was characterized/ classified as $DAPI_{positive}$, $CD45_{negative}$, $CD34_{negative}$, $SOX2_{positive}$/$LIN\text{-}28_{positive}$ and $CK_{positive}$ or $CK_{negative}$. It could be demonstrated that the stem cell associated proteins SOX2 and Lin-28 were expressed in some DTCs BT and AT. Therefore, the development of novel therapeutic strategies targeting these cells might help to improve OS.

Limitations and future investigations

The data presented in this work demonstrate that DTCs with stem cell characteristics actually exist and are able to persist during/after chemotherapy. A larger patient cohort might help to make a more precise and explicit statement about the selection of stem cell marker-positive DTCs during chemotherapy. Since BM aspiration is an invasive intervention and requires local anesthesia the approval of the patient and therefore the availability is limited. Due to the restricted amount of residual material and methodological requirements only two stem cell markers (Lin-28 and SOX2) were analyzed. In future studies, additional stem cell, resistance, dormancy and mesenchymal markers need to be included for a more detailed investigation of the prognostic relevance of these cells.

Since no data about EMT and stem cell associated markers on CTCs/DTCs in ovarian cancer were available, the results within this work allowed further insights into their biological nature and clinical relevance. By analyzing the hematogenous route of metastasis in ovarian cancer, this work elaborated a neglected part in ovarian cancer research and may contribute to a better understanding of the decisive role CTCs/DTC in ovarian cancer progression. The established workflows enable a rapid, cost-efficient and highly sensitive enrichment of CTCs/DTCs with subsequent characterization on the transcriptomic and proteomic level. These assays are the first steps for a detailed recording of molecular ‘snapshots’ of ovarian cancer CTCs/DTCs, before, during and after chemotherapy. In future, they might be correlated with clinically relevant phenotypes and can help physicians to improve therapeutic intervention.

5. Summary

High recurrence rates and chemotherapy resistance imply that ovarian cancer might be initiated and maintained by cancer stem cells. These cells may possess an increased metastatic potential, since they are able to self-renew and insensitive to standard chemotherapeutic agents. Besides that, already during the growth of the primary tumor, the process of epithelial-mesenchymal-transition (EMT) enables tumor cells to detach from the primary tumor by transforming their epithelial phenotype into a mesenchymal one. Ovarian cancer cells, which have undergone this process can either directly enter the peritoneum or transit through the lymphatic or blood system to distant organs. Tumor cells within the blood stream are denoted as circulating tumor cells (CTC) and are able to enter distant organs, e.g. the bone marrow (BM), where they are designated as disseminated tumor cells (DTCs). Both, CTCs and DTCs are supposed to be precursors of subsequent metastatic disease and there is evidence that their presence is correlated with a decreased overall survival (OS) and/or progression free survival (PFS) in ovarian cancer patients.

Consequently, it is useful to detect CTCs/DTCs and to characterize them for EMT and stem cell marker expression. Due to their low frequency compared to numerous blood cells, enrichment of CTCs is necessary to allow their subsequent characterization by molecular methods such as multiplex-RT-PCR. To that aim a typical enrichment strategy for ovarian cancer CTCs (AdnaTest) has initially been used in this project. However, it turned out to be insufficient since it unspecifically co-enriched leukocytes and the subsequent characterization is based on cell pools. For instance co-enriched leukocytes due to their mesenchymal phenotype lead to false positive results regarding expression of EMT-associated transcripts (e.g. neuronal (N)-cadherin and vimentin). Moreover, incompletely differentiated leukocytes disturbed the detection of stem cell associated transcripts (e.g. CD44 (cluster of differentiation 44), ALDH1A1 (aldehyde dehydrogenase 1 family member A1) and Notch1).

To overcome these limitations, a workflow for an optimized isolation and subsequent gene expression profiling of single CTCs from ovarian cancer patients was established. It comprises of a density gradient-based enrichment for nucleated cells, depletion of CD45-positive cells of hematopoietic origin and immunofluorescent labeling of CTCs for the epithelial proteins EpCAM (epithelial cell adhesion molecule) and Muc-1 (mucin-1, cell surface associated). Single CTCs were then isolated by micromanipulation. For subsequent expression profiling of candidate genes a multiplex-RT-PCR was developed which enables the detection of 19 transcripts (4 epithelial, 7 EMT and 8 stem cell associated) simultaneously from one CTC/cell without RNA pre-amplification. Using this approach, the analysis of 77 single OvCar3 cells resulted in heterogeneous gene expression patterns as well as co-expression of several epithelial, EMT and stem cell-associated transcripts, underlining the necessity of single cell analysis. Subsequently, 15 single CTCs derived from three ovarian cancer patients were

characterized: they were positive for stem cell (CD44, ALDH1A1, Nanog, Oct4 (octamer-binding transcription factor 4)) and EMT associated transcripts (neuronal (N)-cadherin, vimentin, Snai2, CD117, CD146). Particularly, inter-cellular and inter-patient heterogeneity as well as co-expression of epithelial, mesenchymal and stem cell associated transcripts within the same CTC were observed.

Analysis of DTCs before (BT) and after (AT) implementation of standard chemotherapy (carboplatin and paclitaxel) was performed on BM aspirates from the iliac crest. DTCs were enriched by density gradient centrifugation and identified by immunocytochemistry. DTCs were detected in 42% BT and in 41% AT. The persistence of DTCs was found in 17% of all patients, 25% were only positive BT, 24% AT and 34% harbored no DTCs. DTC-positive patients (BT) had a significantly decreased OS and were more likely to die than DTC-negative patients (BT). Patients who were initially DTC-negative BT but DTC-positive AT had a significant shorter PFS, while DTC persistence was associated with shorter PFS and OS reaching borderline significance. Immunofluorescent labeling demonstrated that the stem cell associated proteins SOX2 and Lin-28 were expressed in some DTCs BT and AT.

In summary, the newly established workflows within this work enable for a rapid, cost-efficient and highly sensitive enrichment of CTCs/DTCs with subsequent characterization on the transcriptomic and proteomic level for EMT and stem cell associated markers. The presented data illustrate that single-cell analysis in comparison to cell pool analysis can provide new hints about the biology of CTC. Moreover, the results give further insights into the potential clinical relevance of DTCs, which survive chemotherapy and may contribute to ovarian cancer progression. Finally, these assays constitute promising tools to improve ovarian cancer treatment and therapy monitoring.

6. Zusammenfassung

Hohe Rezidivraten sowie Resistenzen gegenüber Chemotherapeutika deuten darauf hin, dass das Ovarialkarzinom durch Krebsstammzellen sowohl initiiert als auch aufrechterhalten werden könnte. Diese Zellen scheinen über ein erhöhtes Metastasierungspotential zu verfügen, da sie in der Lage sind sich selbst zu erneuern und auch unempfindlicher gegenüber Standard-Chemotherapeutika sind. Darüber hinaus ermöglicht der Prozess der Epithelial-mesenchymalen-Transition (EMT) einzelnen Tumorzellen sich bereits während des Wachstums des Primärtumors von diesem zu lösen, da diese in einen mesenchymalen und damit mobileren Phänotyp übergehen. Ovarielle Tumorzellen, die diesen Prozess durchlaufen haben, können anschließend entweder direkt in das Peritoneum vordringen oder durch das lymphatische System bzw. die Blutzirkulation zu/in andere(n) Organen gelangen. Tumorzellen, die sich in der Blutbahn befinden, werden zirkulierende Tumorzellen (ZTZ) genannt und sind in der Lage sich beispielsweise im Knochenmark (KM) anzusiedeln, wo sie als disseminierte Tumorzellen (DTZ) bezeichnet werden. Sowohl ZTZ als auch DTZ werden als Vorläufer einer späteren Metastasierung angesehen und es wurden Hinweise gefunden, die ihre Anwesenheit mit einem verringerten Gesamtüberleben (*overall survival*, OS) und/oder progressionsfreiem Überleben (*progression free survival*, PFS) bei Patientinnen mit Ovarialkarzinom in Verbindung bringen.

Daher ist es sinnvoll, ZTZ/DTZ zu detektieren und hinsichtlich ihrer Expression von EMT- und Stammzell-Markern zu charakterisieren. Aufgrund ihrer geringen Anzahl verglichen mit der Vielzahl von Blutzellen ist eine Anreicherung von ZTZs notwendig, um deren anschließende molekulare Charakterisierung beispielsweise mittels Multiplex-RT-PCR zu ermöglichen. Für diesen Schritt wurde zunächst eine herkömmliche Anreicherungsstrategie für Ovarialkarzinom-ZTZ (AdnaTest) verwendet. Aufgrund einer unspezifischen Co-Anreicherung von Leukozyten und anschließender Charakterisierung des gesamten Zell-Pools stellte sich diese Technik jedoch als unzureichend heraus. So ergaben die unspezifisch angereicherten Leukozyten aufgrund ihres mesenchymalen Ursprungs falsch-positive Ergebnisse für EMT-Transkripte (wie z.B. neuronal (N)- cadherin und Vimentin). Darüber hinaus beeinflussten unvollständig differenzierte Leukozyten den Nachweis stammzell-assoziierter Transkripte (wie z.B. CD44 (*cluster of differentiation 44*), ALDH1A1 (*aldehyde dehydrogenase 1 family member A1*) und Notch1).

Um diese Einschränkungen zu überwinden, wurde ein Arbeitsablauf zur optimierten Isolierung und anschließender Genexpressionsanalyse einzelner ZTZ von Ovarialkarzinom-Patientinnen etabliert – bestehend aus der Anreicherung nukleärer Zellen mittels Dichtegradientenzentrifugation, einer Depletion CD45-positiver Zellen hämatopoetischen Ursprungs und anschließender Markierung der epithelialen Proteine EpCAM (*epithelial cell adhesion molecule*) und Muc-1 (*mucin-1, cell surface associated*) auf der Zelloberfläche von

ZTZ mit Hilfe immunfluoreszent markierter Antikörper. ZTZ wurden dann einzeln mittels Mikromanipulation isoliert. Für die darauf folgende Expressionsanalyse von Kandidatengenen wurde ein Multiplex-RT-PCR entwickelt, mit der 19 Transkripte (4 epitheliale, 7 EMT und 8 Stammzell-assoziierte) gleichzeitig und innerhalb einer einzelnen ZTZ/Zelle gemessen werden können - ohne notwendige Prä-Amplifizierung der RNA. In der Anwendung dieser Methode an 77 einzelnen OVCAR3 Zellen zeigten sich sowohl heterogene Genexpressionsmuster sowie die Co-Expression mehrerer epithelialer-, Stammzell-assoziierter- und EMT-Transkripte, was die Notwendigkeit einer Einzelzellanalyse unterstreicht. Anschließend wurden 15 einzelne ZTZ von drei Ovarialkarzinom-Patientinnen charakterisiert: diese Zellen waren positiv für die Stammzell-assoziierten- (CD44, ALDH1A1, Nanog und Oct4 (octamer-binding transcription factor 4)) sowie die EMT-Transkripte (neuronal (N)-cadherin, Vimentin, Snai2, CD117, CD146). Es zeigte sich sowohl eine interzelluläre als auch eine intertumorale Heterogenität für die analysierten Transkripte. Darüber hinaus konnte beobachtet werden, dass in einer Zelle sowohl epitheliale-, EMT- als auch Stammzell-assoziierte-Transkripte exprimiert wurden.

Ferner wurde die Analyse von DTZ vor (VT) und nach (NT) Standard-Chemotherapie anhand von KM-Aspiraten aus dem Beckenkamm untersucht. DTZ wurden mittels Dichtegradientenzentrifugation angereichert und immunhistochemisch identifiziert. DTZ konnten in 42% der Patientinnen VT und in 41% NT nachgewiesen werden. In 17% aller Patientinnen waren DTZ persistent, 25% waren VT positiv, 24% NT und 34% der Patientinnen waren DTZ-negativ. Zudem hatten DTZ-positive Patientinnen (VT) ein deutlich verringertes OS und starben häufiger als DTZ-negative Patientinnen (VT). Das PFS von Patientinnen, die VT DTZ-negativ aber NT DTZ-positiv waren, war signifikant verkürzt. Die Persistenz von DTZ war mit verringertem PFS und OS, assoziiert – allerdings nur mit *Borderline-Signifikanz*. Unter Verwendung von Immunfluoreszenzfärbungen wurden einzelne DTZs sowohl vor als auch nach Therapie positiv für die Stammzell-assoziierten Proteine SOX2 und Lin-28 getestet.

Zusammenfassend lässt sich sagen, dass die in dieser Arbeit neu etablierten Arbeitsabläufe eine schnelle, kosteneffiziente und hochsensitive Anreicherung von ZTZ/DTZ mit anschließender Charakterisierung auf Transkriptom- und Proteom-Ebene für EMT- und Stammzell-Marker ermöglichen. Die hier präsentierten Ergebnisse verdeutlichen, dass Einzel-Zell Analysen im Vergleich zur Zell-Pool Analyse genauere Aufschlüsse über die spezifische Biologie von ZTZ liefern können. Darüber hinaus erlauben die Daten weitere Einblicke in die potenzielle klinische Relevanz chemoresistenter DTZ, die die Progression des Ovarialkarzinoms möglicherweise fördern können. Schlussendlich sind diese Tests vielversprechende Werkzeuge, um die Behandlung und Therapieüberwachung des Ovarialkarzinoms zu verbessern.

Appendix

A *Statement*

Hiermit erkläre ich, Christina Blassl, dass ich die vorliegende Dissertation selbstständig verfasst und bei keiner anderen Universität bzw. Fakultät in der vorgelegten oder einer ähnlichen Form eingereicht habe. Für die Anfertigung der Dissertation habe ich keine anderen als die angegebenen Hilfsmittel verwendet. Die Stellen, die anderen Arbeiten dem Wortlaut oder dem Sinn nach entnommen sind, wurden unter Angabe der dazugehörigen Quelle kenntlich gemacht.

Düsseldorf, den 07.06.2016

Christina Blassl

B *Abbreviations*

AACR	American Association for Cancer Research
ABC-transporter	ATP-binding cassette transporter
ABCG2	ATP-binding cassette G2
aCGH	Array comparative genomic hybridization
ALDH(1)	Aldehyde dehydrogenase (1)
BM	Bone marrow
Bmi-1	B lymphoma Mo-MLV insertion region 1 homolog
BRCA1/2	Breast cancer 1/2, early onset
CA-125	Cancer antigen-125
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CDH1/2	Cadherin 1/2
CK	Cytokeratin
CNV	Copy number variation
CSC(s)	Cancer stem cell(s)
CTC(s)	Circulating tumor cell(s)
DNA	Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DFS	Disease-Free-Survival
DOI	Digital object identifier
DTC(s)	Disseminated tumor cell(s)
E-cadherin	Epithelial cadherin
e.g.	Exempli gratia
ECM	Extracellular matrix
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
EPCAM	Epithelial Cell Adhesion Molecule
EPISPOT	EPithelial ImmunoSPOT
ER	Estrogen receptor
ERCC1	Excision repair cross-complementation group 1
et al.	*Et alii* (Maskulinum), *et aliae* (Femininum) oder *et alia* (Neutrum)
FDA	Food and Drug Administration
FGF(R)R	Fibroblast growth factor (receptor)
FICTION	Fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasm
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
FISH	Fluorescence in situ hybridization
FN1	Fibronectin-1
HB	Herringbone
HER2	Human epidermal growth factor receptor 2
HPLC	High-Performance Liquid Chromatography
HPSE	Heparanase
HSC(s)	Hematopoietic stem cell(s)
i.e.	Id est
ICC	Immunocytochemistry

IF	Immunofluorescence
IL-6	Interleukin- 6
MET	Mesenchymal–epithelial transition
MGB1	Mammaglobin 1
MMP	Matrix metalloproteinase
MRD	Minimal residual disease
mRNA	Messenger RNA
MSP	Methylation specific PCR
Muc-1	Mucin 1, cell surface associated
Muc-16	Mucin 16, cell surface associated
n	Nano
N-cadherin	Neural cadherin
neg/pos	Negative/positive
NK-cells	Natural killer-cells
NSC(S)	Normal stem cell(s)
NSCLC	Non–small cell lung cancer
Oct-4	Octamer-binding-transcription factor
OS	Overall survival
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reactions
PFS	Progression-free survival
PPIC	Cyclophilin C
PT	Primary tumor
RKI	Robert Koch Institute
RNA	Ribonucleic acid
RNA-ISH	RNA- in situ hybridization
ROS	Reactive oxygen species
RT-(q)-PCR	Reverse transcription-(quantitative)-polymerase chain reaction
SCF	Stem cell factor
SNP	Single nucelotide polymorphism
SOC	Serous ovarian cancer
SOX2	SRY (sex determining region Y)-box 2
TAM(s)	Tumor associated macrphage(s)
TGF-ß	Transforming growth factor beta
TNF	Tumor necrosis factor
TWIST	Twist-related protein 1
upA	Protease urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
vs	versus
WGA	Whole genome amplification
ZEB	Zinc finger E-box-binding homeobox
α	Alpha
β	Beta
µ	Mikro

C *List of figures and tables*

Figures

Tables

D *Wissenschaftlicher Lebenslauf*

Ausbildung

Seit 04/2013	**Forschungslabor der Klinik für Frauenheilkunde und Geburtshilfe der Heinrich-Heine Universität Düsseldorf** Fortsetzung der Promotion; Thema: „Detection and characterization of circulating and disseminated tumor cells in ovarian cancer to improve personalized therapeutic strategies“ Betreuung: (Prof. Fehm/Prof. Neubauer) Angestrebter Abschluss: Dr. rer. nat. Schwerpunkt: Entwicklung von Tumorzell-Anreicherungsmethoden aus dem Blut und Knochenmark sowie Einzelzellanalytik auf RNA- und Protein-Ebene
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Preis(e)

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