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Research Paper

EMT-like circulating tumor cells in ovarian cancer patients are enriched by platinum-based chemotherapy

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ABSTRACT

Background: Assuming that tumor cell dissemination requires a shift to a mesenchymal phenotype, we analyzed the incidence of epithelial-to-mesenchymal-transition (EMT)-like circulating tumor cells (CTCs) in ovarian cancer patients and inquired, how their molecular phenotypes respond to platinum-based chemotherapy and influence outcome.

Results: Before surgery, overall detection rate for epithelial CTCs was 18%. EMT-like CTCs were more frequently observed (30%) and were mutually exclusive to epithelial CTCs in the majority of patients (82%). After chemotherapy, EMT-like CTCs increased up to 52%, accompanied by the "de novo" emergence of PI3Kα+/ Twist+ EMT-like CTCs. Before surgery, PI3K+ EMT-like CTCs in combination with epithelial CTCs indicated decreased OS (p = 0.02) and FIGO I-III patients with residual tumor burden after surgery were more likely to be positive for EMT-like CTCs after chemotherapy (p = 0.02). In the latter group, epithelial CTCs alone significantly correlated with decreased PFS and OS (p = 0.02, p = 0.002), supported by an additional inclusion of PI3K+ CTCs (OS, p = 0.001).

Materials and Methods: Blood samples of 91 ovarian cancer patients before surgery and 31 matched samples after adjuvant chemotherapy were evaluated for CTCs with the AdnaTest *ovarian cancer* and *EMT-1*, analyzing the epithelial-associated transcripts EpCAM, Muc-1 and CA125 and the EMT-associated transcripts PI3Kα, Akt-2 and Twist.

Conclusions: Platinum-based chemotherapy seems to select for EMT-like CTCs in ovarian cancer patients and provokes a shift towards PI3Kα and Twist expressing CTCs, which may reflect clonal tumor evolution towards therapy resistance. It has to be determined, whether this CTC subgroup may serve as a biomarker to identify patients at high risk.

INTRODUCTION

Epithelial ovarian cancer is the fifth leading cause of cancer death among women in Europe and the United States and the second most common gynecological malignancy [1]. Most cases are diagnosed in advanced stages and although response rates to chemotherapy reach up to 80%, the majority of patients cannot be cured. Standard treatment of advanced ovarian cancer is primary surgery aiming at complete resection followed by platinum and paclitaxel based chemotherapy, which has been shown to prolong progression free survival (PFS) as

well as overall survival (OS) [2]. Postoperative residual tumor is one of the most important prognostic factors in advanced ovarian cancer [3–5]. Meanwhile, although new multimodal therapeutic concepts, such as antiangiogenic therapy (e.g. Bevacizumab) or PARP-inhibition (only for recurrent patients), have been designed, more than half of all patients still experience recurrence, resulting in a poor overall prognosis [6–8]. Thus, the identification of innovative therapeutic targets and the identification of predicitive and prognostic biomarker concepts are highly desirable.

In this regard, circulating tumor cells (CTCs) in the blood and disseminated tumor cells (DTCs) in the bone marrow (BM) have already been shown to be promising candidates [9]. Despite the fact that CTCs indicate poor prognosis [10–14], we recently reported that excision repair cross-complementation group 1 protein (ERCC-1)-positive CTCs are present in 8% of patients and constitute an independent predictor, not only for OS but also for PFS. Most interestingly, we discovered the presence of ERCC1^{+}CTC at primary diagnosis to be an independent predictor for platinum-resistance, whereas ERCC1-expression in corresponding primary tumor tissue predicted neither platinum-resistance nor prognosis [15]. Moreover, we reported that the presence of DTCs in the BM, as well as their persistence after platinum based chemotherapy, correlates with poor prognosis and is accompanied by stem cell characteristics of DTCs [16].

The broad heterogeneity of CTCs of cancer patients, including ovarian cancer, has already been demonstrated [17–19] and we may speculate that, besides the presence of epithelial, stem cell-like or potentially platinum-resistant ERCC1-expressing CTCs, some other CTC-phenotypes may play a dominant role for therapy resistance and recurrence in ovarian cancer patients. In this regard, it has been hypothesized that disseminating epithelial cancer cells may undergo a variety of biochemical changes and reversibly acquire fibroblastoid or mesenchymal traits, known as epithelial-to-mesenchymal-transition (EMT), which has already been described for breast cancer CTCs [20, 21]. EMT occurs under physiological conditions, however, is also a key mechanism for malignant progression. “Oncogenic EMT” allows tumor cells to acquire invasive properties and to develop metastatic growth characteristics. Moreover, it protects them from hostile conditions during the dissemination process. Interestingly, disseminated tumor cells may revert to their original epithelial phenotype, referred to as mesenchymal-epithelial-transition (MET), which promotes their colonization and contributes to the establishment of ultimately metastatic sites [22–29].

The PI3K/Akt/mTOR signalling pathway is aberrantly activated in the majority of human malignancies and confers oncogenic functions by promoting proliferation and cell survival [30, 31]. Therefore, this pathway has attracted widespread attention as therapeutic target for several malignancies [32]. Interestingly, recent evidence suggested that the PI3K/Akt/mTOR pathway is also essentially involved in EMT-regulation, thereby promoting tumor aggressiveness [33]. Moreover, repression of the cellular adhesion molecule E-cadherin, which is considered a hallmark of EMT, is mediated by EMT-associated transcription factors, such as Twist, Snail, Slug or Zeb [34]. Of those, especially Twist raised considerable attention, since it can also mediate invasiveness, drug resistance and EMT through a positive feedback loop with Akt, therefore providing a direct link to the PI3K/Akt/mTOR pathway [33].

In ovarian cancer, aberrant activation of the PI3K/Akt/mTOR pathway has also been reported and EMT is supposed to promote chemo-resistance [30, 35]. Interestingly, it has been suggested that cisplatin treatment of ovarian cancer cells generates residual cells with EMT-like traits [36]. However, it is still actively discussed, whether ovarian cancer cells in the primary tumor actually undergo a complete transition to a mesenchymal state [37]. Moreover, it is unknown, whether EMT-associated phenotypes extend to CTCs in the blood of ovarian cancer patients and whether they contribute to the heterogeneity of ovarian cancer CTCs. Therefore, assuming that dissemination of ovarian cancer cells requires at least a partial shift to a mesenchymal phenotype, the main purpose of our study was to analyze the incidence of epithelial and EMT-like CTCs at primary diagnosis of ovarian cancer. Moreover, we investigated how their detection rate is influenced by platinum-based chemotherapy. As a secondary objective, we analyzed EMT-associated transcript markers in more detail and were interested, how particular molecular phenotypes of EMT-like CTCs respond to platinum-based chemotherapy.

RESULTS

Platinum-based chemotherapy selects for EMT-like CTCs in ovarian cancer

We analyzed the epithelial associated marker transcripts EpCAM, Muc-1 and CA-125 as well as the representative EMT-associated marker transcripts PI3Kα, Akt-2 and Twist before surgery (n = 91) and in paired blood-samples after platinum-based chemotherapy (n = 31). Positivity for each CTC-subtype was defined by the detection of at least one of the transcripts of each marker panel, respectively. Epithelial CTCs were detected with an overall incidence of 18% before surgery, which slightly decreased to 14% after platinum-based chemotherapy. EMT-like CTCs were observed with a considerably higher detection rate at baseline (30%) and their incidence further increased after chemotherapy to an overall detection frequency of 52% (Figure 1, Supplementary Table 1), suggesting that platinum-based chemotherapy selects for EMT-like CTCs.

Epithelial and EMT-like CTCs exhibit a low phenotypic overlap

Having described the incidence of epithelial and EMT-like CTCs by separate analyses, we now investigated the overlap between epithelial and EMT-associated phenotypes (Figure 2, Supplementary Table 1). Before surgery, patients with detectable CTCs were positive for either EMT-associated transcripts (58%) or epithelial-associated transcripts (24%). Thus, epithelial and EMT-like CTCs were mutually exclusive in the majority of patients (82% in total). Interestingly, only a minor fraction of patients showed up with dual positivity of epithelial and EMT-like CTCs (18%), indicating that epithelial- and EMT-like CTCs seem to represent mostly independent CTC populations with low phenotypic overlap. After chemotherapy, this trend was retained and the proportion of exclusively EMT-positive CTCs further increased up to 76%, whereas the number of patients with exclusively epithelial CTCs or dual positivity each declined to 12%.

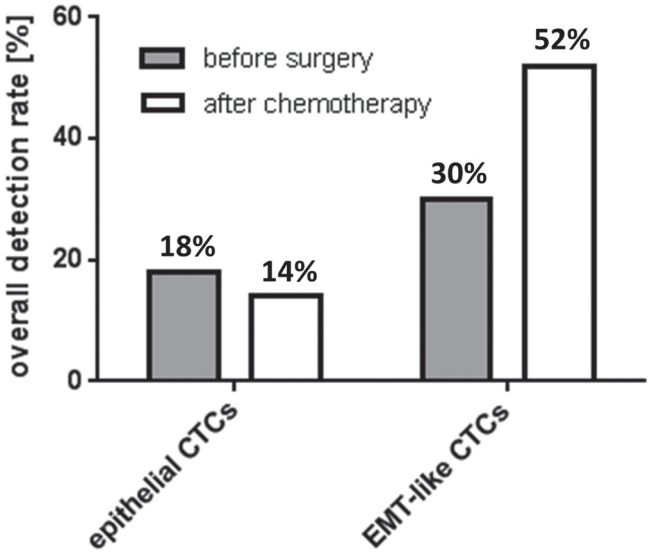

Figure 1: Overall detection frequency of epithelial and EMT-like CTCs in ovarian cancer. The bar chart illustrates overall detection rates of epithelial and EMT-like CTCs in ovarian cancer patients before surgery and after chemotherapy. Percentages for the two classes of CTCs were calculated independently from each other and, in both cases, refer to the whole study population (before surgery: $n = 91$, after chemotherapy $n = 31$). A patient was considered "epithelial CTC-positive" or "EMT-like CTC-positive", if at least one of the epithelial markers or one of the EMT-associated markers was detectable, respectively.

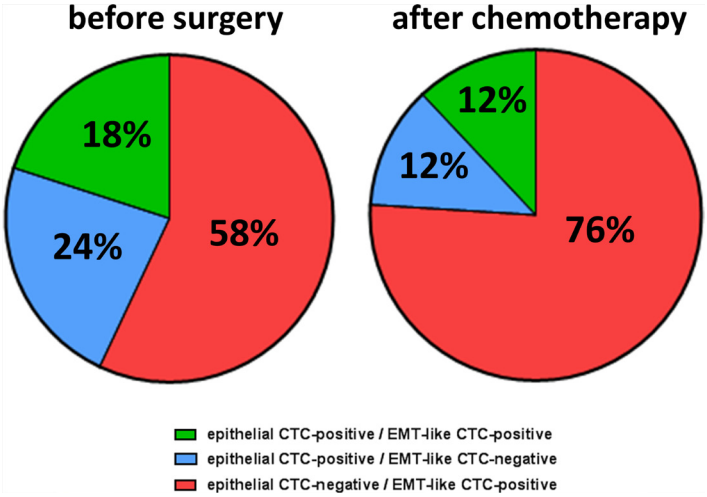

Figure 2: Phenotypic overlap of epithelial and EMT-like CTCs. The pie chart depicts the overlap of epithelial and EMT-like CTCs in ovarian cancer patients before surgery and after chemotherapy. Percentages were calculated in reference to all patients with overall CTC-positivity. Besides patients with exclusively epithelial (blue) and exclusively EMT-like CTCs (red), there were also patients, harbouring both CTC populations in their blood (green).

PI3Kα and Twist positive EMT-like CTCs are specifically enriched by platinum-based chemotherapy

We were further interested in the molecular phenotypes of epithelial and EMT-like CTCs in ovarian cancer and their response to platinum-based chemotherapy. Figure 3A illustrates the marker distribution among epithelial CTCs. Detection frequencies for EpCAM and Muc-1 were calculated independently from each other and in reference to only those patients with positivity for epithelial CTCs. Before surgery, positivity for epithelial CTCs mostly resulted from Muc-1 positivity, which was detected in 81% of cases, whereas EpCAM transcripts were less frequently detected (38%). After chemotherapy, detection frequency was slightly reduced for both marker transcripts; however, Muc-1 positivity remained three times more abundant than EpCAM-positivity (75% Muc-1-positive vs. 25% EpCAM-positive, Supplementary Table 1).

Figure 3B depicts the molecular heterogeneity of epithelial CTCs in more detail. Percentages were calculated in reference to only those patients with positivity for epithelial CTCs, now also considering dual positivity. Before surgery, the majority of epithelial CTCs were exclusively Muc-1 positive (62%), whereas only 19% were exclusively EpCAM-positive and further 19% showed dual positivity for Muc-1 and EpCAM. After chemotherapy, dual EpCAM+Muc-1-positive CTCs were no longer detectable and only Muc-1 (75%) or EpCAM (25%) positive CTCs were observed. CA-125 transcripts could not be detected at any time in our patient cohort, indicating expression levels below the detection limit of our assay or complete absence of CA-125 transcripts in the enriched CTC populations (Supplementary Table 1).

Subsequently, we performed the same kind of analysis for the EMT-associated marker panel (Figure 3C). Here again, percentages for PI3Kα, Akt-2 and Twist were calculated independently from each other in reference to only those patients with positivity for EMT-like CTCs. Before surgery, PI3Kα was observed in 35% of patients with positivity for EMT-like CTCs and Akt-2 in 46% of patients. Twist was most frequently detected (54%, Supplementary Table 1). Interestingly, after platinum-based chemotherapy, positivity rates for PI3Kα and Akt-2 slightly decreased, whereas Twist positivity was substantially elevated up to 69% in post-therapeutic blood samples, which is in accordance to the overall increase of EMT-like CTC (Figure 1).

Figure 3D depicts the molecular heterogeneity of EMT-like CTCs in more detail. Percentages were calculated in reference to only those patients with positivity for EMT-like CTCs, now also considering dual or triple positivity. Before surgery, exclusively Twist positive CTCs were most abundant (42%) followed by Akt-2 (19%) and PI3Kα-positive CTCs (12%). Additional CTC-phenotypes with dual or triple positivity for EMT-markers were observed with low or moderate detection frequency (15% PI3Kα+Akt-2 / 4% Akt-2+Twist / 8% PI3Kα+Akt-2+Twist). After chemotherapy, selective changes in the composition of molecular CTC-phenotypes became obvious. Interestingly, an additional molecular CTC-phenotype with dual PI3Kα+Twist positivity emerged, mostly, at the expense of PI3Kα+Akt-2-positive CTCs (Supplementary Table 1). The proportion of the other CTC-phenotypes remained largely stable in response to platinum-based chemotherapy. Notably, while the proportion of exclusively Twist or exclusively PI3Kα positive CTCs also remained nearly unchanged in post-chemotherapeutic blood samples, we conclude that the increase in the overall incidence of EMT-like CTCs (as shown in Figure 1) is accompanied by the "de novo" emergence of a dual PI3Kα+Twist positive CTCs after chemotherapy.

Clinical relevance of EMT-like CTCs

We inquired, whether the presence and enrichment of EMT-like CTC subtypes correlates with the patient's clinicopathological parameters or with their survival. We observed the trend that patients with a residual tumor burden after primary debulking surgery were more likely to have EMT-like CTCs in their blood after adjuvant chemotherapy than patients with a macroscopically complete tumor resection. This association became statistically significant, after excluding patients with distant metastasis (FIGO IV; $p = 0.02$).

Subsequently, we investigated prognostic significance of epithelial and EMT-like CTCs before surgery and after chemotherapy by Kaplan-Meier analysis. In the unselected total patient cohort, no prognostic relevance of epithelial CTCs before surgery or after chemotherapy could be demonstrated (Supplementary Figure 3). However, after excluding patients with FIGO IV as possible confounders, which per se have a poor prognosis, the presence of epithelial CTCs at primary diagnosis significantly indicated decreased PFS (HR: 2.63, 95% CI: 1.24–15.53; $p = 0.027$) and OS (HR: 5.79, 95% CI: 2.98–162.7; $p = 0.003$, Figure 4A, 4B). There was no prognostic significance of EMT-like CTCs before surgery or after chemotherapy in the total patient cohort (Supplementary Figure 4). However, combined analysis showed that the presence of epithelial CTCs or PI3Kα transcripts indicates reduced OS in the total study population (HR: 3.25, 95% CI: 1.31–15.47; $p = 0.018$, Figure 4C). Interestingly, this finding could be confirmed with increased statistical significance, after excluding FIGO IV patients, and the presence of epithelial CTCs or PI3Kα transcripts at primary diagnosis indicated reduced PFS (HR: 2.35, 95% CI: 1.06–8.74; $p = 0.042$) and OS (HR: 7.22, 95% CI: 3.21–111.5; $p = 0.001$, Figure 4D, 4E).

DISCUSSION

In the present investigation, we analyzed incidence and molecular phenotypes of EMT-like CTCs in the blood of ovarian cancer patients and monitored their response to platinum-based chemotherapy. EMT-like CTCs were, already at primary diagnosis, more abundantly detected than epithelial CTCs and showed low phenotypic overlap with epithelial CTCs. After chemotherapy, we observed a selective enrichment of EMT-positive CTCs, which was accompanied by the "de novo" emergence of dual PI3Kα and Twist positive CTCs.

At present, there is no standard definition for the identification of CTCs and a variety of CTC-enrichment and detection strategies are available, based on CTC-associated surface antigens or intrinsic (physical or tumor-biological) CTC properties, such as size, deformability, invasive capacity or telomerase activity [38, 39]. Subsequent CTC-detection can be carried out by a broad spectrum of methods, such as immunocytological or molecular biology based assays [10, 11, 39–43]. In line with our previous investigations [10, 15], we took advantage of the AdnaTest *OvarianCancer*, which allows a more detailed molecular characterization of enriched CTCs. Notably, overall detection frequency of epithelial CTCs at primary diagnosis, reported herein, was comparable to our previous analyses, confirming comparability of the underlying study with our previous observations on larger patient cohorts [10]. In the present investigation, we were interested in the incidence and dynamics of EMT-associated CTC-phenotypes, which, to the best of our knowledge, has never been investigated in ovarian cancer patients so far.

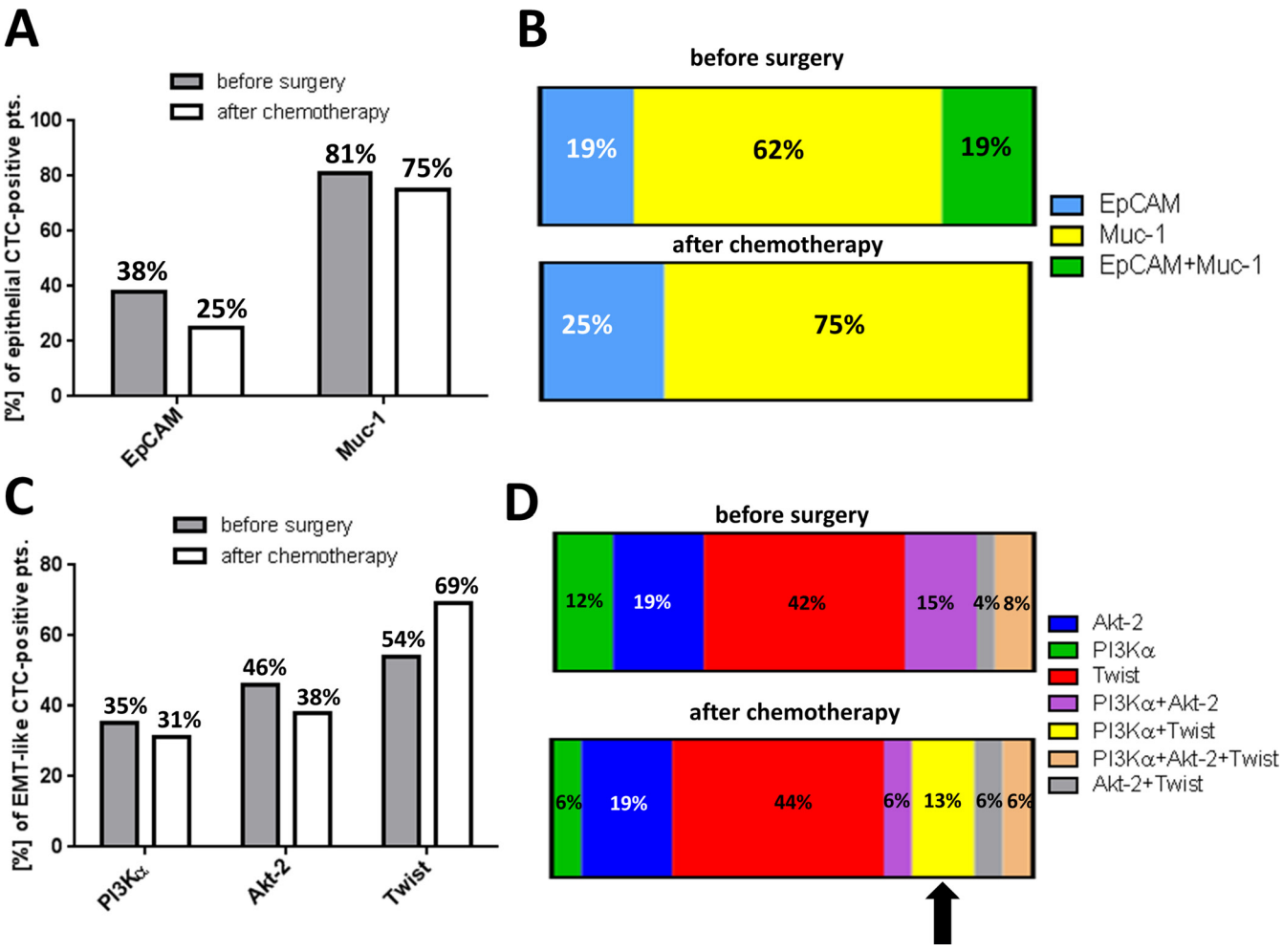

Figure 3: Heterogeneity of EMT-associated CTC-phenotypes and their response to platinum-based chemotherapy. The figure summarizes molecular phenotypes of EMT-like CTCs and their response to platinum-based chemotherapy. (**A**) The bar chart shows the marker distribution in epithelial CTC-positive patients. Percentages for EpCAM and Muc-1 were calculated independently from each other and in reference to only those patients with positivity for epithelial CTCs. (**B**) The stacked bar chart illustrates the marker distribution of epithelial CTC-positive patients, also considering dual-positivity for EpCAM and Muc-1. (**C**) The bar chart shows the marker distribution in EMT-like CTC-positive patients. Percentages for PI3Kα, Akt-2 and Twist were calculated independently from each other and in reference to only those patients with positivity for EMT-like CTCs. (**D**) The stacked bar chart illustrates the marker distribution in EMT-like CTC-positive patients, now also considering dual- or triple positivity for EMT-associated transcripts.

We observed that EMT-associated marker transcripts considerably expanded the phenotypic range of CTC-detection. Already at primary diagnosis, EMT-like CTCs were more abundant than epithelial CTCs. This is in accordance with findings on breast cancer, reporting that EMT is a rare event in the primary tumor, however, frequently occurs among CTCs [21, 44]. Therefore, we conclude that EMT is also a common event among ovarian cancer CTCs and might already have been initiated in the primary tumor. Interestingly, recent data from a pancreatic cancer mouse model even suggested that EMT-like CTCs can already be shed into the circulation from pre-invasive lesions [45].

We explicitly used the term EMT-"like" CTCs in our study and strictly avoiding any other descriptive terms that might imply that these CTCs already acquired a fully mesenchymal state. Since the immunomagnetic enrichment of CTCs in our assay is based on the epithelial surface epitopes Muc and EpCAM, this assay cannot detect fully mesenchymal CTCs, which have completely downregulated their epithelial surface epitopes. Thus, EMT-like CTCs, selected and characterized herein, express the epithelial marker proteins EpCAM and Muc on their surface, allowing immunomagnetic selection, however, do not express EpCAM or Muc-1 on transcript level above the detection limit of our assay. At a first glance, this may appear counterintuitive, however, discordances between protein and transcript expression profiles of a cell can be explained by post-transcriptional modifications of messenger RNA or differences in the half-live time between messenger RNA and their corresponding proteins [46–48]. Notably, since these CTCs also co-express Akt-2, PI3Kα or Twist, we describe snapshots of "semi-mesenchymal" CTCs [49]. Semi-mesenchymal CTCs could be either on their way to an ultimately mesenchymal phenotype (EMT) or on their way back to an epithelial phenotype (MET) or, alternatively, they could persist in this intermediate state. Although the biology of semi-mesenchymal CTCs is largely unknown, we could speculate that particularly a semi-mesenchymal state reflects an aggressive CTC-phenotype with high degree of plasticity, which facilitates the adaption of CTCs to hostile environmental stimuli during dissemination. This is in line with the hypothesis, that EMT (and its reversion MET) is a highly dynamic process and describes different continuous phenotypes, rather than a dichotomous switch between epithelial and mesenchymal states. Notably, those phenotypic changes directly influence the yield of CTC-detection assays that are based on epithelial selection markers [49]. However, our assumption of continuous CTC phenotypes in ovarian cancer is not necessarily supported by the fact that we observed a trend for a mutual exclusion between patients with only epithelial CTCs and those with only semi-mesenchymal CTCs. This is a very

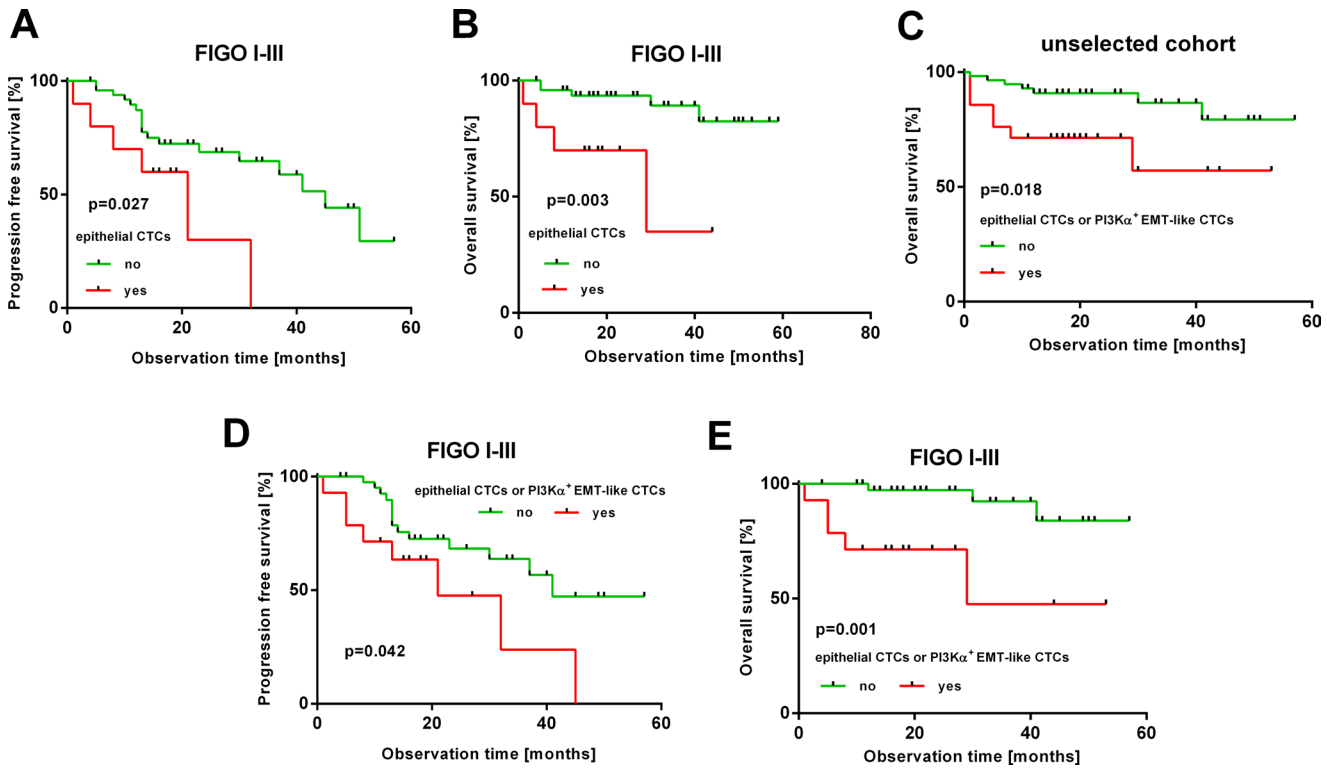

Figure 4: Prognostic relevance of epithelial and EMT-like CTCs. The Kaplan-Meier plots show prognostic relevance of different CTC-subtypes at primary diagnosis. (**A, B**): epithelial CTCs in FIGO I-III patients (thus without distant metastasis) (**C**): epithelial or PI3Kα-positive CTCs in the unselected patient cohort (**D, E**): epithelial or PI3Kα-positive EMT-like CTCs in FIGO I-III patients. Red curves represent patients, positive for the respective CTC-subtype(s); green curves represent patients, negative for the indicated CTC-subtype(s).

interesting finding and may indicate that there is also a subgroup of ovarian cancer patients with "fully" epithelial CTCs, without any shifts towards EMT. These CTCs may have entered the bloodstream via passive dissemination [50], however, clinical relevance of this finding requires further investigation.

Applying our EMT-associated marker set, we revealed a heterogeneous spectrum of EMT-associated CTC-phenotypes, pointing to CTC-heterogeneity in ovarian cancer, which has already been reported for CTCs in a variety of other cancer entities, such as prostate or breast cancer [51, 52]. Notably, we cannot distinguish with our assay, whether a co-expression of more than one marker-transcript is derived from CTCs, actually co-expressing these markers on a same cell, or from separate semi-mesenchymal CTC-populations, concomitantly present in the "pool" of enriched CTCs.

Oncogenic EMT has received considerable attention over the past years and is associated with cancer aggressiveness, metastasis and tumor cell plasticity [29]. Interestingly, we observed a clear increase of semi-mesenchymal CTCs in response to platinum-based chemotherapy, due to a shift towards PI3Kα and Twist expression. This finding is of particular clinical interest, since it has already been described that cancers may acquire resistance to systemic treatment as a result of clonal evolution and selection [53]. We therefore assume a clonal selection of CTCs with activated PI3Kα and Twist associated signaling pathways, which might be therapy refractory and could be responsible for recurrence of ovarian cancer. This assumption is strongly supported by our previous studies on breast cancer patients, in which we already showed that preferentially semi-mesenchymal and potentially platinum-resistant (ERCC1-expressing) CTCs remain after neoadjuvant chemotherapy [54]. Moreover, a further independent key publication reported on dynamic changes in the epithelial and mesenchymal composition of breast cancer CTCs in response to therapy. In this study, clinical response was accompanied by a switch to predominantly epithelial CTCs, whereas progressive disease correlated with the increase of mesenchymal CTC-phenotypes [21]. For ovarian cancer, *in vitro* experiments already suggested that cisplatin treatment of ovarian cancer cells generates residual cells with EMT-like traits [36]. Therefore, we assume that PI3Kα and Twist positive CTCs may reflect tumor evolution in response to platinum-based chemotherapy. Interestingly, this also suggests a link to recent studies on genomic tumor evolution, reporting on an increase in activating PIK3CA mutation among cell free tumor DNA of breast cancer patients, following paclitaxel treatment [53]. In this context, our findings could have several diagnostic or therapeutic implications, since PI3Kα and Twist are functionally involved in pathways controlling tumor cell survival or platinum-resistance [55, 56]. The most important limitation of our study is the small number of patients, particularly when comparing the detection frequency of single transcript markers in pre- and post-therapeutic blood samples. Nevertheless, this detailed analysis, albeit only descriptive, was considerably informative for us and complemented our key finding, the enrichment of semi-mesenchymal CTC after platinum-based chemotherapy.

Among our exploratory survival analysis, we did not confirm prognostic relevance of epithelial CTCs in the unselected study population, which is in contrast to our previous finding and could be explained by the yet limited follow-up period of the present study [10]. Nevertheless, after excluding FIGO IV patients as possible confounders, prognostic relevance of epithelial CTCs could be restored. Apart from this, the presence of epithelial CTCs at primary diagnosis, in combination with PI3Kα-positivity, indicated poor prognosis not only in FIGO I-III patients, but also in the unselected cohort, suggesting that PI3Kα-positivity marks a clinically relevant subgroup of EMT-associated CTCs, which could be derived from the untreated primary tumor. In contrast, we reported that patients with residual tumor after primary debulking were more likely to be positive for EMT-like CTCs after adjuvant chemotherapy, indicating that this CTC-population could be disseminated directly from residual tumor burden under the selective pressure of chemotherapy, after conversion to a semi-mesenchymal state.

Conclusively, this is the first study on EMT-like CTCs in ovarian cancer, reporting that platinum-based chemotherapy provokes a shift of molecular phenotypes towards PI3Kα and Twist expressing CTCs, which may reflect clonal tumor evolution. Therefore, we encourage to further investigate the functional role of semi-mesenchymal CTCs in the malignant progression of ovarian cancer and to determine, whether these EMT-like CTCs can be a biomarker for high risk minimal residual disease in ovarian cancer. In this context, an extended multi-marker panel with mesenchymal and tumor stem cell-associated genes, which we recently established for metastatic breast cancer [18], could be a useful liquid biopsy tool, in order to detect a broad spectrum of CTC-phenotypes for therapy monitoring. Moreover, PI3Kα and Twist positive CTCs could be an attractive therapeutic target, since PI3K/Akt/mTOR pathway inhibitors are currently being investigated for ovarian cancer among several preclinical studies and also a few ongoing clinical trials [32] (NCT01623349, NCT02476955). Moreover, it was shown for ovarian cancer that low dose metformin, a first line drug for treating diabetes, can also reduce the expression of EMT-associated makers, such as Twist, suggesting further options for potentially targeting this CTC-population [57]. Since the biology of ovarian cancer CTCs is largely unknown, we believe that our finding could be a step forward in understanding their heterogeneity and their dynamics in response to chemotherapy.

Table 1: Patient characteristics at the time of primary diagnosis

Total	95
Age	median 61 years, (31–82) years
FIGO stage	
I–II	16 (17%)
III	61 (64%)
IV	18 (19%)
Nodal status	
No	33 (37%)
N1	32 (43%)
Nx	30 (20%)
Grading	
I–II	32 (43%)
III	63 (57%)
Residual tumor	
Macroscopic complete resection	48 (51%)
Any residual tumor	40 (42%)
Unknown	7 (7%)
Histologic type	
Serous	81 (80%)
Mucinous	5 (14%)
Other	9 (6%)
Survival	
PFS 1	median 6 months, (0–51 months)
OS2	median 21 months, (1–59 months)
Alive	15 (16%)
Dead	72 (76%)
Unknown	8 (8%)
Recurrence	
No relapse	50 (43%)
Relapse	28 (55%)
Unknown	17 (2%)

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

MATERIALS AND METHODS

Patient characteristics

The present study was conducted at the Departments of Gynecology and Obstetrics at the University Hospitals of Essen and Dresden, Germany. In this study, a total of 95 patients, diagnosed between 2010 and 2014 with histologically confirmed epithelial ovarian cancer, were analyzed. Clinical characteristics of the patients are documented in Table 1. Informed written consent was obtained from all patients and the study was approved by the Local Ethic Committees (Essen 05–2870; Dresden EK 236082012) and was performed according to the declaration of Helsinki. 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 [58] and tumor staging was classified according to the Fédération Internationale de Gynécology et d'Obstétrique. The whole study population underwent primary radical surgery. Total abdominal hysterectomy, bilateral salpingo-oophorectomy, infragastric omentectomy and peritoneal stripping was performed. The most important aim of surgery was to achieve macroscopic complete tumor resection. Radical pelvic and para-aortic lymphadenectomy were only performed if macroscopic complete tumor resection was achieved. All patients received at least six cycles of carboplatinum AUC 5 and paclitaxel 175 mg/m^2.

Enrichment and molecular characterization of ovarian cancer CTCs

Peripheral blood (5 ml) from each patient was collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) and processed within 4h for the enrichment of CTCs and subsequent expression analysis, according to the AdnaTest *OvarianCancer Detect* and the AdnaTest *EMT-1 Detect* (QIAGEN, Hilden, Langenhagen, Germany, Supplementary Figure 1, 2). These assays have been described in detail elsewhere [10, 15, 44]. The Adnatest was performed in biological replicates; therefore two independent consecutive blood samples were obtained from each patient at each time point. Briefly, we applied immuno-magnetical enrichment of CTCs (Adnatest *OvarianCancer Select,* Adnatest *EMT-1 Select),* targeting epithelial cellular adhesion molecules.

For the detection of epithelial CTCs, RNA was isolated and gene expression analysis was performed by reverse-transcription (RT) and multiplex RT-PCR, detecting EpCAM, Muc-1, and CA-125 (AdnaTest *OvarianCancer Detect)*. In this assay, amplicons with the following sizes were generated: EpCAM: 396bp; Muc-1: 293bp; CA-125: 432bp. For the detection EMT-like CTCs, RNA was isolated and gene expression analysis was performed by reverse-transcription (RT) and multiplex RT-PCR, detecting PI3Kα, Akt-2 and Twist (AdnaTest *EMT-1 Detect)*. Contaminating leukocytes (about 1500 per sample) were reduced by approximately 10fold using a special washing buffer (*AdnaWash* buffer) enabling the proper differentiation of EMT expression profiles with a specificity and sensitivity of > 90%, which was confirmed in healthy donor samples [20, 44]. In this assay, amplicons with the following sizes were generated: PI3Kα: 595bp, Akt-2: 306 bp; Twist: 203bp. β-actin served as an internal control (amplicon size: 119 bp) and PCR-products were visualized with the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA). An amplicon concentration of > 0.2 ng/µl was applied as threshold for EpCAM, Muc-1 or CA-125 positivity. Amplicon concentration of > 0.2 ng/µl was applied as threshold for Akt-2, > 0.25 ng/µl for PI3Kα and > 0,15 ng/µl for Twist positivity, respectively.

The analytical sensitivity of the detection of CTC-associated EMT-transcripts was determined by the analysis of a low number of target cells (5 IGROV ovarian cancer cells spiked into 5 ml blood of healthy donors). Healthy donor samples without spiked tumor cells were used to determine the specificity of the test. Applying the above mentioned amplicon cut-off values, 97% of 30 healthy donor samples were negative for EMT markers. These experiments demonstrate that a potential risk of false-positive events is negligible in our present analysis.

Abbreviations

BM: bone marrow; CTC(s): circulating tumor cell(s); DTC(s): disseminated tumor cell(s); EMT: epithelial-to-mesenchymal-transition; EpCAM: epithelial cell adhesion molecule; ERCC1: excision repair cross-complementation group 1; MET: mesenchymal-to-epithelial-transition; Muc-1: Mucin-1; OS: overall survial; PFS: progression free survival; RT: reverse transcription.

Authors' contributions

IC, SKB, PB, PW, SH, RK and JDK made substantial contributions to the conception and design of the study, to the experimental work or to the acquisition of data and to the analysis/interpretation of the results. JDK, SKB, IC, PW and SH were involved in drafting the manuscript or revising it. All authors read and approved the manuscript in its final version.

CONFLICTS OF INTEREST

Sabine Kasimir-Bauer is a consultant for QIAGEN, Hilden, Germany.

Siegfried Hauch is an employee of QIAGEN, Hilden, Germany.

All other authors declare that they have no conflict of interest.

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