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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).