Tumorigenicity of Ascites-Derived Tumor Cells: Insights Into the Molecular Mechanisms of Ovarian Cancer Progression and Therapy Resistance

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Research

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Abstract

Background: Ovarian cancer (OvCa) cells disseminate primarily intraperitoneally. Here, detached tumor cell aggregates (spheroids) from the primary tumor are generally regarded as “metastatic units”, which exhibit a survival benefit, probably due to the protective microenvironment and their unique molecular characteristics. Hence, current therapeutic concepts such as classical chemotherapy are not sufficient preventing growth and spread of OvCa spheroids.

Methods: In the current study we analyzed the cellular composition of ascites from ovarian cancer patients using flow cytometry and the tumorigenic potential of the different subpopulations in an intraperitoneal mouse model. Comparative transcriptome analyses (RNAseq) from ascites-derived tumor cells spheroids (n=10) vs. tumor samples from different metastatic sites (n=30) were further performed in order to identify key molecular players responsible for the special cellular characteristics of OvCa spheroids.

Results: In vitro culture of ascites-derived cells gave rise to two different subpopulations: an adherent cell population (ADs) including mainly CD90+ cells with highly proliferative rates in vitro but no tumorigenic potential in vivo, and a non-adherent cell population (NADs) containing principally EpCAM+/CD24+ cells with low proliferative potential in vitro. NADs included cell aggregates and single cells, the first showing a high content (> 80%) of tumor cells (EpCAM+/CD24+). Enriched tumor cell spheroids from the ascites using cell strainers showed higher tumorigenic potential in vivo in comparison to the original ascites-derived cell population. Interestingly, the different metastatic spread patterns observed in the mice resembled the tumor dissemination pattern found in the corresponding patients. RNAseq analyses from tumor-spheroids revealed up-regulation of genes involved in chemoresistance (TGM1, HSPAs, MT1s), cell-adhesion and cell barrier (PKP3, CLDNs, PPL) and the oxidative phosphorylation (OXPHOS) process compared to the solid tumor tissue samples. Additionally, down-regulation of extracellular matrix components and angiogenesis-related genes could be observed. Targeting OXPHOS by metformin treatment led to reduced viability of ascites-derived spheroids from OvCa patients, showing to some extent a synergistic effect with cisplatin treatment.

Conclusions: the actual study contributes to a better understanding of the biology of ovarian cancer spheroids and to the identification of new treatment opportunities in advanced ovarian cancer.

Background

Ovarian cancer is the most lethal gynecological malignancy, with an expected 5-year mortality rate > 60%. This low survival rate, that has modestly improved in the last decades, is mainly caused by often late disease detection. As ovarian cancer is asymptomatic at an early stage, around 70% of all diagnosed patients already show peritoneal metastasis at diagnosis (1). Disseminated ovarian cancer cells spread primarily within the peritoneal cavity, where small tumor deposits can be detected frequently in the omentum and peritoneum. Unlike other solid cancers, ovarian cancer rarely disseminates through the
bloodstream, although pelvic and para-aortic lymph nodes are often involved. Frequently, patients develop ascites, a pathological fluid within the abdominal cavity containing tumor cells but also cells from no tumorigenic origin and diverse soluble factors that create a favorable environment for tumor growth and dissemination. Tumor cells within the ascites are present either as single cells or more frequently as aggregates or so-called floating spheroids (2, 3), the last representing the main source for peritoneal metastasis. Also, in advanced ovarian cancer patients without ascites, tumor cell spheroids are also present in the peritoneal lavage fluid collected during surgery. Recently, using an \textit{in vivo} ovarian cancer model, it could be shown that tumor spheroids mainly arise from multicellular detachment from the primary tumor rather than from single cells aggregating within the abdominal cavity. More precisely, cell aggregates in ascites arise from collective dissemination of neighboring cells in the primary tumor and in turn single spheroids developed individual metastatic sites (4). These observations are in line with the genetic clonal mapping of disseminated ovarian cancers in patients. Here, phylogenetic analysis of multiple intraperitoneal sites from individual patients showed that most metastatic sites were phylogenetically pure (5). Tumor cells within cell spheroids exhibit a survival advantage in comparison to single floating cells due to the protective microenvironment created by cellular interactions with other tumor and non-tumoral cells. Recent in vivo data suggest that tumor-associated macrophages (TAMs) may promote spheroid formation and tumor growth. Here, TAMs were found within the spheroids, thereby secreting large amounts of epidermal growth factor (EGF), which leads to upregulation of integrin and intercellular adhesion molecule 1 (ICAM-1) expression in tumor cells (6). Furthermore, spheroids represent a more chemoresistant population since chemotherapeutic drugs show a lower incorporation and poor diffusion in such multicellular structures (7, 8). Moreover, it has been suggested that chemoresistance is caused, in part, by tumor cells entering a non-cycling state with low metabolic activity, which is a characteristic feature of detached tumor cells and spheroids floating in the peritoneal fluid or ascites (3).

In conclusion, ovarian carcinomas are highly heterogeneous tumors and tumor cell aggregates (spheroids) that detach from the primary tumor comprise unique clones, which might or might not survive in the peritoneal fluid. We assume that those spheroids that persist and are able subsequently to attach to the peritoneal cavity can be considered as “metastatic units”. These cellular structures share certain biological characteristics that might be useful in the development of new therapeutic strategies.

In the present study we aim to characterize the cellular composition and the tumorigenic potential of the different subpopulations found in the ascites fluid of ovarian cancer patients. Further, a first insight into the specific molecular characteristics of tumorigenic ascites-derived tumor cell spheroids was accomplished by RNAseq analyses.

\section*{Materials And Methods}

\section*{Patient material}

Ascites was collected from patients diagnosed with advance ovarian cancer at the University Medical Centre Hamburg-Eppendorf between 2017 and 2020. Ascites was obtained during debulking surgery from
patients with primary and recurrent disease. Detailed patient characteristics are presented in supplementary table 1. All patients gave written informed consent to access their biomaterial and review their clinical records according to our investigational review board and ethics committee guidelines (#190504 and PV6012). Clinical parameters were retrieved from a detailed institutional database providing information on clinicopathological factors, surgical and therapeutic procedures.

**Preparation, cultivation of ascites-derived cells and isolation of ascites-derived spheroids**

Ascites or lavage from advanced ovarian cancer patients were centrifuged at 1200 rpm for 5 minutes at room temperature. Supernatants were collected and frozen down at -20 °C for other purposes. Cell pellets were resuspended in RBC lysis buffer (Red Blood Cell lysis buffer, Invitrogen, San Diego, CA, USA) and incubated for 15 minutes at room temperature. After 5 minutes centrifugation at 1200 rpm, the cell pellets were washed with PBS (Phosphate Buffered Saline solution, Sigma-Aldrich, St. Louis, Missouri, USA) and resuspended in PBS. 10 µl of cell suspension were mixed with 10 µl of a 0.04% trypan blue solution and cell amount, cell size and aggregation status were observed under the microscope.

Ascites-derived cells were cultured in MCDB medium (MCDB 105 Medium and Medium 199 (1:1) supplemented with 10% fetal calf serum (FCS), and penicillin/streptomycin (2 mM) (1%) (Life Technologies, CA, USA) or in the clarified supernatant of the ascites at 37 °C on low attachment plates in the presence of 5% CO₂ and 95% humidity. Here, some cells floated as spheroids or single cells in the medium (non-adherent cells, NADs) while some cells attached to low attachment plates (adherent cells, ADs).

For some experiments, large-sized cell aggregates present in the ascites-derived pellet were separated by using 15 µm cell strainers (pluriSelect, Leipzig, Germany). Briefly, the cell pellet was resuspended in PBS and put on the top of the cell strainer softly shanking until no fluid in the upper part was observed. Additional 5–10 ml PBS were added to the cell strainer to flush and wash the cells. The fluid containing all cells < 15 µm were collected in a 50 ml tube. Subsequently the cell strainer was turned upside down and cells and cell aggregates retained in the strainer were flush back onto a new 50 ml tube using 5–10 ml PBS.

**In vivo intraperitoneal mouse model**

Ascites-derived cells (aprox. 3 × 10⁶ cells) from the original ascites cell pellet or from the separated tumor spheroid fraction were resuspended in MCDB medium without FCS (200 µl) and injected into the peritoneal cavity of immunodeficient female mice (CB17/lcr-Prkdcscid/lcrIcoCrl (SCID) or C.129S6(B6)-Rag2tm1Fwa N12 (Rag2-Model 601), as previously described (9). Due to the limited number of ascites-derived cells available after preparation, habitually one mouse was injected per patient sample. For some patient samples, injections with cells corresponding to different size fractions were performed. In total 30 mice were included in this study. The animals were housed with a 12 h day-night cycle in a temperature- (21 °C) and humidity- (50%) controlled room. All mice were kept in individually ventilated cages under pathogen-free conditions, fed with sterile standard food and water ad libitum. Mice that showed strong
signs of tumor progression (ascites, shaggy coat and loss of appetite; (10)) were anesthetized with xylazine/ketamine (120/16 mg/kg body weight, Bayer, Leverkusen, Germany/ Graeub, Bern, Switzerland) and sacrificed after terminal cardiac blood collection by cervical dislocation. The dissemination pattern found was documented, tumors at the injection site, metastases and lungs were excised, frozen or formalin-fixed and embedded in paraffin. Animal experiments were conducted according to the UKCCCR guidelines for the welfare of animals in experimental neoplasia (10). The mouse experiments were approved by the local licensing authority (Freie und Hansestadt Hamburg, Behörde für Gesundheit und Verbraucherschutz, Amt für Verbraucherschutz, project #G16/55).

Results

Characterization of cellular components in ascites from ovarian cancer patients

The ascites or lavage from OvCa patients (n = 141) were collected during debulking surgery. After the first centrifugation step, only samples containing a visible cell pellet were morphologically assessed by phase contrast microscopy immediately after collection on day0 (n = 75). In 60% of the ascites samples we found both single cells and cell aggregates (spheroids) as shown exemplary in Fig. 1 (original fraction), whereas the rest of the samples showed essentially a single cell population. To characterize the cellular components of ascites-derived cells (n = 37), FACS (Fluorescence-activated Cell Sorting) analysis was performed with an established antibody panel including CD45 (immune cells marker), CD24/EpCAM (tumor markers) and CD90 (mesothelial-like cell marker) at day0. These 37 samples measured by FACS included 5 low grade and 32 high grade ovarian cancer samples, different FIGO stages (IB: n = 1, IIIB: n = 5, IIIC: n = 2 and IV: n = 8) as well as 4 recurrent tumors. After excluding dead cells, a high heterogeneity regarding the content of CD45+, CD90+, CD24+ and EpCAM+ cells was observed among samples (n = 37). The tumor cell population, defined as EpCAM+ strongly varied from 0.8–99.8% among the ascites samples, whereas the range of immune and mesenchymal-like cells was between 2.3–95.8%, respectively.

Further, cell strainers of 15 µm were used to separate the original pellet into a small and a big cellular fraction. The multicellular aggregates (spheroids) were mainly collected in the big fraction, while the cell population smaller than 15 µm, defined as small fraction, included only single cells (Fig. 1). After this filtering step the CD24+/EpCAM+ cell population was strongly enriched in the big fraction compared to the original and small pellet, while most CD45+ and CD90+ cells were found in the small fraction, which contained a relatively low amount of CD24+ and/or EpCAM+ single cells. Figure 1 displays representative FACS analysis showing a clear reduction of immune (CD45+: from 72–21%) and mesenchymal-like cells (CD45-/CD90+: from 51–13%) in the big fraction and a remarkably increase of ovarian cancer cells (CD45-/EpCAM+ and CD45-/CD24+: 43–91% and 49–92%, respectively). The purity of the cell spheroids enriched in the big fraction could be corroborated by ICC and IF analysis. Figure 2 displays exemplary pictures from 3 samples showing cell aggregates with strong cellular EpCAM and a
few immune cells (CD45+). Additionally a strong E-Cadherin staining was found in two samples by ICC. In line with the FACs analysis, sample #7 showed in the IF a high content of EpCAM-positive cells and a lack of CD90-positive cells.

**Cellular characterization of different subpopulations arising from ascites-derived cells** **in vitro**

Ascites-derived cells (day0) were cultured in low-attachment plates with MCDB medium for up to two weeks. Here, two different cell populations could be distinguished after approx. 5 days cultivation time: adherent elongated spindle-shaped mesenchymal-like or cobblestone-shaped epithelial-like cells (adherent cells, ADs) and multicellular aggregates (spheroids) and small single cells floating in the supernatant without attachment (non-adherent cells, NADs) (Fig. 3A). Both cell populations showed a remarkably different proliferative behavior. ADs were typically highly proliferative, whereas the NAD population remained quiescent but viable for up to two weeks cultivation time. In order to better characterize these two cell populations, an additional FACs panel including a tumor marker (EpCAM), stem cell markers (CD44, CD24 and CD133) and mesenchymal-like and mesothelial cell markers (CD90, podoplanin and mesothelin) was established. Thus, the AD population (n = 9) exhibited a high content of CD90+, podoplanin + and to some extent mesothelin + and CD44 + cells, whereas NADs (n = 9) were mainly EpCAM + and CD24+ (Fig. 3B and Supplementary Fig. 1). Figure 3B displays representative FACs results from sample #15 showing a strong CD90, podoplanin, and CD44 positivity for AD cells, whereas the corresponding NAD cell population only express EpCAM and CD24. Additional FACS analyses from six AD/NAD pairs are shown in the supplementary Fig. 1.

Remarkably, 2 samples showed a different behavior **in vitro** (Supplementary Fig. 2). They both contained low proliferative AD cells with an additional strong EpCAM, CD24, CD133 staining, suggesting a cancer stem cell phenotype.

These findings led us to the conclusion that in most of the ascites-derived samples the highly proliferative population of adherent cells (ADs) does not comprise ovarian cancer cells but rather a mesenchymal-like cell population and cells from mesothelial origin. To prove this assumption, the tumorigenic potential of these cells were further tested in vivo using an intraperitoneal mouse model.

**In Vivo Tumorigenicity Of Ascites-derived Cells**

In a next step we were interested in the tumorigenicity of the different populations found in the ascites-derived cells **in vivo**. Therefore, ADs, spheroids (cell fraction size > 15 um), single cells (cell fraction size < 15 um) and/or original pellets from different ovarian cancer patients (n = 21) were each intraperitoneally injected in immunodeficient mice. None of the mice injected with ADs (n = 7) developed peritoneal carcinomatosis, whereas injection of the original ascites cell-pellets, without cell separation or previous cultivation, led to tumor or ascites formation in 50% and 20% of the injected samples, respectively. Similarly, all spheroid samples (n = 9) gained from the original pellet via filtration with a cell strainer developed in a time period ranging from 18 to 56 weeks metastatic ovarian cancer, thereby showing
different extents of dissemination and patterns (Fig. 4). For two cases, the corresponding single-cell fraction was injected in parallel. Here, no tumor formation could be observed, even though this fraction contains single tumor cells, as showed before. Interestingly, we found a strong similarity in the tumor dissemination pattern observed in mice and in the corresponding ovarian cancer patient, as described in the surgery protocol. In our mouse model, we could clearly distinguish between a miliary-like dissemination pattern found in 11 samples and a “non-miliary” tumor spread generated by 2 samples, the last characterized by one or two big tumor bulks within the peritoneal cavity or retroperitoneal located (n = 2). One sample developed only malignant ascites, containing big amounts of tumor cell spheroids without any solid tumor lesion. Remarkably, reinjection of mouse ascites-derived tumor cells led to tumor and to some extent to ascites development in all cases (n = 2), thereby maintaining the same spread pattern and showing faster progression rates.

**Transcriptome Of Tumor Cell Spheroids From Ovarian Cancer Patients**

As shown in the *in vivo* model, ascites-derived tumor cell spheroids from ovarian cancer patients can be considered “metastatic units” that promote peritoneal carcinomatosis as well as retroperitoneal tumor cell dissemination. We assume that tumor cell spheroids biologically differ from those tumor cells in the primary or metastatic tumor tissue, since they must be able to survive as floating units and eventually to attach at the metastatic site. In order to identify the molecular players responsible for these specific biological characteristics, the transcriptome of ascites-derived tumor spheroids and tumor tissue samples were compared. RNAseq analysis from 10 different tumor spheroid patient samples and 30 tumor tissue samples, including primary and metastatic tissue, were performed. Here, the ascites-spheroid (A1-A10) and the tumor sample group (T1-T30) form two distinct clusters in the principal component analysis (PCA; Fig. 5A). Subsequently, comparative expression analyses between the ascites group and three different tumor groups, each one containing 10 tumor samples, were carried out (Fig. 5B). The tumor tissue samples included in each of the three groups were selected based on the patient characteristics (FIGO, age, histology, grading, tumor stage and lymph node status) to resemble the ascites group. Only those genes commonly and significantly (|Log2-fold change| > 1 and FDR < 0.1) de-regulated in the ascites-derived spheroids in comparison with all three tumor groups were further evaluated and are displayed in the supplementary tables 2 and 3. A selected group of these genes has been presented in the heat map on Fig. 5. Interestingly, the oxidative phosphorylation pathway, including a large number of genes, i.e. ATPsynthases, NADH oxidoreductases and cytochrome c oxidases, are significantly up-regulated (p value: 5.6952e-12, FDR: 1.6539e-8) in the ascites spheroids in comparison to the tumor cells from the primary or metastatic tissue. Higher mRNA levels of genes related to chemoresistance (i.e. TGM1, heat shock proteins and metallothioneins), to cell-cell adhesion and also barrier molecules (i.e. PKP3, PPL, CLDN4/7 and filaggrin) were found in ascites spheroids vs. tumor tissue as well. Three glycosylation enzymes, NEU1, B4GALT5 and CHST4 as well as several transcription factors (FOS, JUN and KLF4/6) were also up-regulated in the ascites group. Interestingly, the increased expression of CD163
and MARCO (macrophage receptor with collagenous structure) in the group of ascites-derived spheroids, suggest an important role of macrophages on the biology of these cellular structures. Among the 1316 significantly down-regulated genes in the ascites spheroids, two main pathways: angiogenesis and extracellular structure organization could be identified, which are significantly down-regulated (p value and FDR are almost 0). Numerous genes involved in angiogenesis (i.e. ANGTP2, APLNR, PDGFRβ or the cytokines CCL11 and CCL2) and as expected in the extracellular structure organization (i.e. several collagen proteins, MMP16/19, fibronectin, lumican, versican, and the hyaluronan synthase 2).

**Inhibition of the OXPHOS pathway via metformin treatment in tumor spheroids from ovarian cancer patients**

In order to evaluate the effect of an OXPHOS pathway inhibition on ascites-derived tumor spheroids from ovarian cancer patients, *in vitro* cell viability was measured after treatment with metformin alone as well as in combination with cisplatin. Here, 5 samples from different ovarian cancer patients were incubated with increasing metformin (1 and 5 mM) and cisplatin concentrations (1, 5 and 10 µg/ml) for 48 hours. Cell viability was assessed subsequently using CellTiter-Glo solution as described in the methods section. Among the 5 samples analyzed, 3 samples showed a strong response to the metformin treatment, even at the lowest concentration of 1 mM. In these cases the simultaneous treatment with metformin and cisplatin led to a significant viability reduction when compared with each treatment alone. One sample (#11) showed no response to metformin, whereas it was highly sensitive to cisplatin treatment. Interestingly, one sample (#15) from a recurrent ovarian cancer patient showed as expected no response to cisplatin treatment, but a significant reduced cell viability after 48 treatment with 1 and 5 mM metformin. The simultaneous treatment with cisplatin and metformin led surprisingly to an even stronger cytotoxic effect on this ovarian cancer sample (Fig. 6).

**Discussion**

In ovarian cancer, detached single and tumor cell aggregates (spheroids) from the primary tumor that persist in the peritoneal fluid represent the main source of intraperitoneal metastasis (14). Specially, tumor cells within the spheroids exhibit a survival benefit and may represent a key element of chemotherapy-sensitive recurrence. In the present study we were able to identify and enrich this tumorigenic subpopulation within the ascites of ovarian cancer patients and further elucidate via RNAseq analysis unique molecular characteristics of these cellular structures.

Malignant ascites itself constitutes a favorable milieu for tumor cells to progress. It contains soluble factors such as cytokines, chemokines, growth factors, and extracellular matrix fragments as well as a complex mixture of cells including tumor, stromal cells and infiltrating immune cells [2]. The cellular part of ascites includes single cells and cell aggregates, so-called floating spheroids [5, 6]. In the present study, we could show that the single cell population includes some tumor cells, but it is principally composed of immune cells and to a less extent of a mesenchymal-like cell population, defined in our analysis by a CD90 positivity. In contrast, the cell aggregates found in most ovarian cancer samples contained a much
higher (> 80%) percentage of tumor cells showing a strong EpCAM expression or combined EpCAM and CD24 positivity. Here, the tumor purity as well as the protective environment created by these structures may explain the high rate of successful tumor development observed, when injecting tumor-spheroids intraperitoneally in immunodeficient mice, in contrast with single cell tumor injections. Moreover, the interaction between tumor cells and other cellular components within the spheroids seems to be essential in order to keep their compact structure, but it also enhances the survival ability and invasive potential of the tumor cells. In this context, different cell populations such as cancer-associated fibroblasts (CAF clandest). In glioblastoma, MARCO-expressing TAMs induce a phenotypic shift towards mesenchymal cellular state of glioma stem cells, promoting both invasive and proliferative activities, as well as therapeutic resistance to irradiation (24). Additional analysis are required in order to elucidate the impact of MARCO-positive TAMs on spheroid tumor cells and whether this interaction might further influence disease progression of ovarian cancer patients.

One characteristic feature of tumor spheroids is their low chemosensitivity, in part attributed to a low proliferative profile (3). The standard chemotherapy for ovarian cancer patients, consisting in a paclitaxel and carboplatin combination, selectively targets and eliminates highly proliferative tumor cells (25). In poorly vascularized tumor areas however, cells become quiescent (26) and in turn less responsive to therapy. A recent study has estimated that in ovarian cancer spheroids more than 60% of the cells are quiescent (4). Moreover, it has been described that quiescent tumor cells use preferentially the mitochondrial OXPHOS pathway for their ATP production (27). In line with this data, our RNAseq analysis revealed a significant up-regulation of the OXPHOS pathway in the tumor-spheroids isolated from the ascites of ovarian cancer patients compared to corresponding solid tumor tissue samples. Thus, OXPHOS pathway inhibition opens an attractive therapeutic window for the specific target of tumor spheroids, as the major vehicle of peritoneal metastasis in OvCa. By using OXPHOS inhibitors, spheroid tumor cells might not be able to cover their high ATP demand. Contrary to normal cells that can activate glycolysis in response to OXPHOS inhibition, quiescent tumor cells within the spheroids have no access to sufficient glucose in order to compensate the loss of ATP production and might die (28). Interestingly, several drugs, including metformin, that have been used clinically for non-oncologic indications have
emerged as effective OXPHOS inhibitors (29). Several cohort studies have described a protective effect and an association of metformin with longer overall survival in ovarian cancer patients (30–33). Also, two *in vitro* studies have found reduced ovarian cancer cell proliferation, migration and increased apoptosis (34) as well improved sensitivity in drug-resistant ovarian cancer cell lines (35) after metformin treatment. In contrast, a recent meta-analysis that excluded studies considered to have the potential for immortal time bias, suggested no overall survival benefit associated with use of metformin (36). A recent pilot study evaluating the efficacy of metformin plus first-line chemotherapy versus chemotherapy alone in a small cohort of ovarian cancer patients found no effects of metformin, neither(37) Our in vitro analyses might explain these contradictory findings. Here, ascites-derived tumor-spheroids from different ovarian cancer patients showed in the majority of the samples a strong effect of metformin alone as well as in combination with cisplatin treatment, on the tumor cell viability, including a cisplatin-resistant tumor from a recurrent ovarian cancer patient. One sample, however, showed no response to metformin even at high concentrations, indicating that inhibition of the OXPHOS pathway might be not a universal target for all ovarian cancer patients. Ongoing analyses in our group aim the identification of the specific molecular features that might discriminate metformin responders from non-responders.

Our RNAseq data has further revealed several factors up-regulated in spheroids that are linked to cell chemoresistance. The transglutaminase 1 (TGM1), an enzyme that is mainly found in the epidermis, catalyzes protein bonds, so-called cross-linking, which give the tissue strength and stability. In gastric carcinoma TGM1 has been shown to promote the stem cell character and chemoresistance of tumor cells via modulation of the Wnt/beta-catenin signalling pathway. Further, several members of the heat shock protein 70 family (Hsp70) were found to be significantly up-regulated in the tumor spheroids compared with the tumor tissue. The human Hsp70 family consists of eight highly homologous members of chaperone molecules that differ in their intracellular localization and expression pattern. Specially, HSPA1A/1B and HSPA6, which code for the proteins Hsp70 and Hsp70-6, respectively are only expressed at low or undetectable levels under physiological conditions, but are rapidly induced by cellular stress (38). In cancer cells, the effect of Hsp70 has been not only related to its chaperone activity, but rather to its antiapoptotic role and the regulation of cell signaling. In ovarian cancer, increased Hsp70 expression was found in chemoresistant cells. Here, Hsp70 proteins block the translocation of Bax into the mitochondria and the release of mitochondrial proteins into the cytosol (39). Additionally, three metallothioneins (MT1E, MT1M and MT1X) were highly up-regulated in the spheroids. MTs are small cysteine-rich proteins with a key role in metal homeostasis and protection against heavy metal toxicity. Consequently, a drug resistance function has been described in the context of cancer (40), though specifically in ovarian cancer no difference between MT expression in tumors from chemotherapy-treated vs. untreated patients could be found (41). Still, MT expression has been negatively associated with survival time in primary ovarian carcinomas (42).

Besides the low proliferative rate and chemosensitivity, tumor cells within the spheroids might acquire specific adhesive characteristics that support a protective and compact cellular aggregation structure (43). In this context, our RNAseq analysis revealed high mRNA levels of integrin α3 (ITGA3), Claudins 4 and 7 (CLDN4/7), desmosome proteins plakophilin 3 (PKP3) and periplakin (PPL) as well as the barrier
protein filagrin (FLG), the last showing an aprox. 10fold up-regulation in the ascites-derived spheroids compared with tumor tissue. Interestingly, recent data raised the possibility that molecules with mechanical barrier function may be used by cancer cells to protect them from immune cell infiltration and immune-mediated destruction. Here, authors identified eight genes, including PPL and PKP3, whose increase expression in human melanoma metastases and ovarian cancers was associated with a lack of Th1 immune signatures and further strongly correlated with shorter overall survival (44).

The in vitro and in vivo behavior of ascites-derived cells has been reported by other groups before (2, 43, 45, 46). In our study we showed similar results as previously described, namely in the majority of the samples ascites tumor spheroids from ovarian cancer patients showed a quiescent and non-adhesive phenotype when cultured in vitro, whereas the single cells gave rise to an adherent and highly proliferative population. In contrast, intraperitoneal injection in immunodeficient mice showed just the opposite picture; namely tumor development was observed in most of the spheroid samples, but none of the ADs developed carcinosis. FACS and ICC analyses revealed an explanation for this contrary behavior, showing that the spheroids consist principally of tumor cells population, whereas the single cells were mainly of non-epithelial origin. We assume the lack of an adequate stimulus in vitro prevent tumor spheroids to attach and further spread, thereby highlighting the key role of the intraperitoneal environment for tumor progression in ovarian cancer. Thus, the key role of fibroblasts, immune, adipocytes, mesothelial and endothelial cells for disease progression has been broadly described in the last years (47, 48).

Remarkably, two samples showed a totally different pattern regarding their cellular distribution and in vitro behavior. Here, the AD populations included a high percentage of tumor cells, as they showed a strong staining for EpCAM. These results emphasizes the high heterogeneity of “ovarian cancer” and the need to decipher the different biological subtypes behind this entity, in order to develop specific and targeted therapies. In this context, the ascites-derived tumor spheroids might represent a suitable model to address this question. In the present study we could show that ascites-derived spheroids from ovarian cancer patients clearly depict the biology of the individual disease, especially if we consider that their dissemination pattern in the mice clearly mimic the one observed in the patient.

Moreover, we could identify several molecular players that might help us to better understand the biology of ascites-derived tumor spheroids. Ongoing analysis in our group aim to prove the functionality of these markers and to explore their role as potential therapeutic targets.

**Abbreviations**

OvCa  
Ovarian cancer  
ADs  
Adherent cells  
NADs  
Non-adherent cells
CD
Cluster of Differentiation

EpCAM
Epithelial cell adhesion molecule

TGM1
Transglutaminase 1

HSPAs
Heat shock protein family A

MT1s
Metallothioneins 1

PKP3
Plakophilin 3

CLDNs
Claudins

PPL
Periplakin

OXPHOS
Oxidative phosphorylation

TAMs
Tumor-associated macrophages

EGF
Epidermal growth factor

ICAM-1
Intercellular adhesion molecule 1

RBC
Red blood cells

PBS
Phosphate Buffered Saline solution

FCS
Fetal Calf Serum

SCID
Severe combined immunodeficiency

UKCCCR
United Kingdom Coordinating Committee of cancer Research

FFPE
Formalin-fixed paraffin-embedded tissue

DAB
3,3'-diaminobenzidine

DAPI
4',6-diamidino-2-phenylindole
DNBseq
DNA Nanoballs sequencing

KEGG
Kyoto Encyclopedia of Genes and Genomes

FACS
Fluorescence-activated Cell Sorting

FIGO
International Federation of Gynecology and Obstetrics

PCA
Principal component analysis

FDR
False discovery rate

ATP
Adenosine triphosphate

NADH
Nicotinamide Adenine Nucleotide

NEU1
Neuraminidase 1

B4GALT5
Beta-1,4-galactosyltransferase 5

CHST4
Carbohydrate sulfotransferase 4

KLF
Kruppel like factor

MARCO
Macrophage receptor with collagenous structure

ANGPT2
Angiopoietin 2

APLNR
Apelin receptor

PDGFRβ
Platelet derived growth factor receptor beta

CCL
C-C motif chemokine ligand

MMP
Matrix metallopeptidase

ITGA3
Integrin subunit alpha 3

FLG
Filaggrin
Declarations

Ethics approval and consent to participate

All patients gave written informed consent to access their biomaterial and review their clinical records according to our investigational review board and ethics committee guidelines (#190504 and PV6012) in the University Medical Centre Hamburg-Eppendorf.

Animal experiments were conducted according to the UKCCCR guidelines for the welfare of animals in experimental neoplasia. The mouse experiments were approved by the local licensing authority (Freie und Hansestadt Hamburg, Behörde für Gesundheit und Verbraucherschutz, Amt für Verbraucherschutz, project #G16/55).

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

YD performed the experiments, analyzed data and contributed to write the manuscript. VL contributed to the animal experiments. KL contributed to the production of the RNASeq data. MYQ analyzed the RNASeq data. BS provided patient material and characteristics. CS and US designed the mouse experiments. LOF conceptualized the project, designed the experiments, and was a major contributor in writing the manuscript. All authors read, reviewed and approved the final manuscript.

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Figures

![Figure 1](image)

Representative analysis on ascites-derived cells from one patient (#10). Pictures (left side) display the morphology of ascites-derived cells in culture before (original pellet) and after separation into small and big fractions.
big fractions using a 15 μm strainer. Corresponding FACS results show the percentage of immune cells (CD45+), mesenchymal-like cells (CD90+), and tumor cells (EpCAM+ / CD24+) in each population.

Figure 2

Immunocytochemistry and immunofluorescence analysis of spheroids from ovarian cancer patient ascites. (A) Paraffin embedded spheroids from two different patients (#5 and #16) showing strong EpCAM and E-Cadherin expression and a few CD45+ cells. (B) IF staining of spheroids from patient #7 showing a similar pattern, namely a high percent of EpCAM+ and few CD45+ cells embedded in the aggregate structures.
Figure 3

In vitro behavior of ascites-derived cells. (A) Representative pictures of ascites-derived cells from two ovarian cancer patients after five days in vitro culture. Two different cell populations can be observed, adherent elongated spindle-shaped mesenchymal-like or cobblestone-shaped epithelial-like cells (adherent cells, ADs) and multicellular aggregates (spheroids) and small single cells floating in the supernatant without attachment (non-adherent cells, NADs). (B) Representative FACs analysis of ADs and corresponding NADs from sample #15 showing strong CD90/Podoplanin/CD44 expression and EpCAM and CD24 expression, respectively.
Figure 4

In vivo behavior of ascites-derived cells. (A) Number of ascites-derived samples analyzed in vivo: i.p. injection of original ascites cell-pellets (n=10) led to tumor formation in 4 cases and in 2 of them additionally to ascites formation. All spheroid samples (big fraction, n=9) developed metastatic ovarian cancer. All reinjected samples developed peritoneal carcinosis as well. (B) Three main dissemination patterns were observed, a miliary-like, a non-miliary like and solely ascites. The metastatic spread pattern in the mice resembled the tumor dissemination pattern in the corresponding patient.
Figure 5

RNAseq analysis of ascites-derived spheroids. (A) PCA Plot showing a clear separation of the ascites-spheroid samples (A1-A10) and the tumor sample group (T1-T30). (B) Hit maps displaying selected up-regulated and down-regulated genes in the spheroid tumor cells in comparison with the tumor cells from primary or metastatic tissue samples.
Figure 6

Inhibition of the OXPHOS pathway via metformin treatment in tumor spheroids from ovarian cancer patients. Five samples from different ovarian cancer patients were incubated with increasing metformin (1 and 5 mM) and cisplatin concentrations (1, 5 and 10 μg/ml) for 48 hours and cell viability was subsequently assessed. Three samples showed a strong response to the metformin treatment and simultaneous treatment with metformin and cisplatin led to a significant viability reduction when compared with each treatment alone. Sample #11 showed no response to metformin, whereas it was highly sensitive to cisplatin treatment. Sample #15 from a recurrent ovarian cancer patient showed as expected no response to cisplatin treatment, but a significant reduced cell viability after 48 treatment with 1 and 5 mM metformin.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.pptx
- SupplementaryFigure2.pptx
- SupplementaryTable1.xlsx
- SupplementaryTable2Upregulatedgenes.xlsx
- SupplementaryTable3Downregulatedgenes.xlsx