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Review Article

Ascites modulates cancer cell behavior, contributing to tumor heterogeneity in ovarian cancer

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Malignant ascites constitute a unique tumor microenvironment providing a physical structure for the accumulation of cellular and acellular components. Ascites is initiated and maintained by physical and biological factors resulting from underlying disease and forms an ecosystem that contributes to disease progression. It has been demonstrated that the cellular contents and the molecular signatures of ascites change continuously during the course of a disease. Over the past decade, increasing attention has been given to the characterization of components of ascites and their role in the progression of ovarian cancer, the most malignant gynecologic cancer in women. This review will discuss the role of ascites in disease progression, in terms of modulating cancer cell behavior and contributing to tumor heterogeneity.

Epithelial ovarian cancer (EOC) is the most lethal gynecologic cancer and is very heterogeneous malignancy. EOC are diverse not only in histopathology, but also in etiology, and are mostly diagnosed in the late stages.[1] This fatal disease is inherently silent, with patients often presenting with fluid accumulation in the abdominal cavity, called ascites, at diagnosis. The standard treatment of EOC includes cytoreductive surgery followed by chemotherapy with carboplatin and paclitaxel. Despite initially high response rates to this standard treatment, most patients develop recurrent disease and the ascites is present in almost all recurrences.[2] The presence of malignant ascites correlates with deterioration in quality of life and with a poor prognosis.[3,4]

The importance of the tumor microenvironment in cancer progression has been increasingly recognized and it plays an essential role in mediating and sustaining the hallmarks of cancer. In particular, ascites is gaining recognition as a unique form of tumor microenvironment responsible for hallmark of ovarian cancer. The link between the presence of ascites and ovarian cancer progression was first proposed by Lopez *et al.*[5] Since then, numerous studies have contributed to the characterization of the ascites components, further revealing it's key roles in the hallmarks of ovarian cancer.

To overcome the limitations of current anticancer agents, better understanding of EOC and its tumor microenvironment is needed. Remarkable progress has been made in research on malignant ascites, expanding our knowledge of both the cellular (tumor cells and stromal cells) and acellular (soluble factors) components (Fig. 1). All of these components work in coordination to create tumor-friendly microenvironments, which fosters the acquisition of hallmarks. Here, we discuss the current understanding of the roles of ascites components in EOC progression, with specific emphasis on the individual key factors contributing to tumor heterogeneity.

Ascites as a tumor microenvironment in ovarian cancer

Ovarian cancer is characterized by rapid growth and spread of intraperitoneal tumors and patients present with a huge amount of ascites in the peritoneal cavity.[6] Ascites provide local tumor microenvironment and is composed of both cellular and acellular factors, which modulate cancer cell behavior and contribute to tumor heterogeneity in ovarian cancer. Their functional contributions are discussed below (Fig. 2).

Components of ascites. *Cellular components.* The origin and phenotype of the cells in the ascites is poorly understood. Similar to other tumor microenvironments, the cellular components of ascites contain a complex heterogeneous mixture of cell populations, including tumor cells and stromal cells, each with

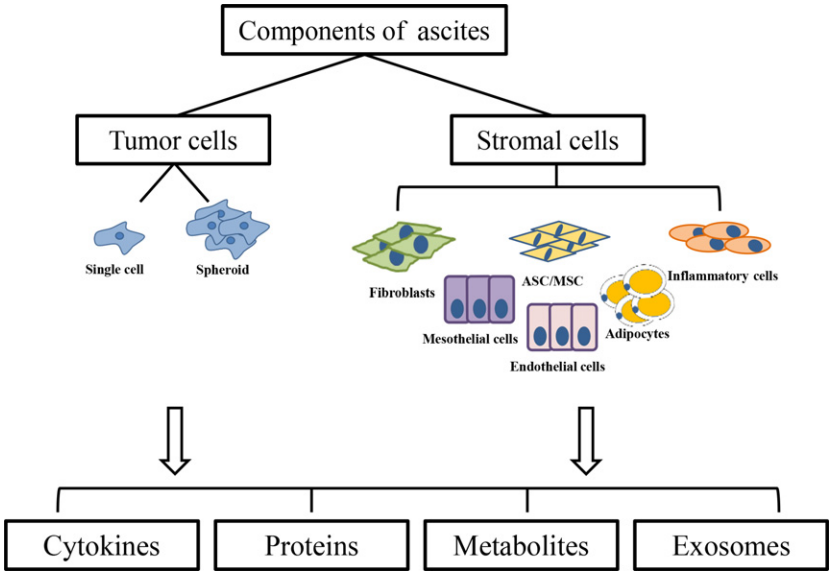

Fig. 1. Overview of cellular and acellular components of ascites. Ascites is composed of both tumor cells present either as single cells or as spheroids and stromal cells, including fibroblasts, mesothelial cells, adipose tissue derived stromal cells (ASC/MSC), endothelial cells, adipocytes and inflammatory cells. These cellular components communicate with each other through acellular factors, including cytokines, proteins, metabolites and exosomes.

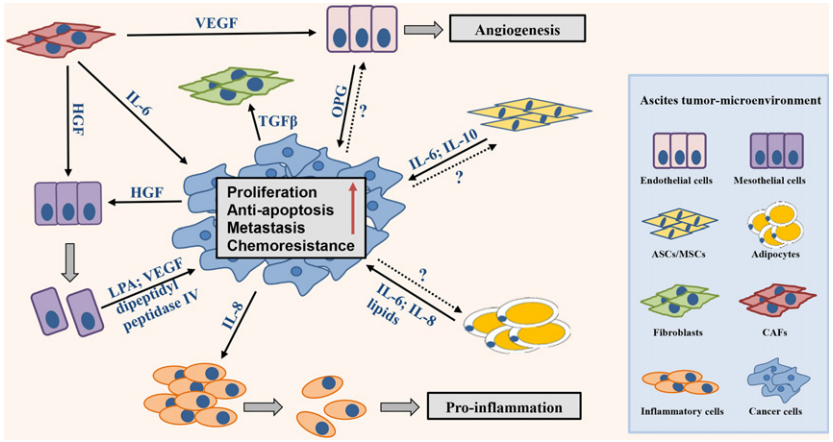

Fig. 2. Schematic overview of the signaling interactions during malignant progression in ascites. The arrows in bold indicate which acellular components are used to communicate between cellular components. Unknown communications are indicated with a dashed arrow. See text for description of individual components and their functional contributions in ovarian cancer.

a defined role. The stromal cellular components of ascites include fibroblasts, endothelial or mesothelial cells, adipocytes, adipose tissue-derived stromal cells, bone marrow-derived stem cells and immune cells.[7,8] Some of these stromal cellular components show abnormal features, including activation of growth and angiogenesis.[9,10] In several tumors, stromal cells play a significant role in malignant progression. In particular, the malignant role of cancer-associated fibroblasts (CAF) has been highlighted, through autocrine-paracrine loops, and promoting proliferation, migration and invasion of cancer cells. CAF secrete factors that can transduce signals to cancer cells as well as to themselves, establishing reciprocal reinforcement of growth and migration signals as well as chemoresistance.[11] Similarly, the interactions between EOC cells and human peritoneal mesothelial cells (HPMC) in ascites are believed to be important for tumor progression.[9,12] Like CAF, HPMC secrete factors that promote tumor growth. For example, lysophosphatidic acid (LPA) is produced by immortalized HPMC and has been shown to enhance adhesion, migration and invasion of ovarian cancer cells.[12] These cancer-associated mesothelial cells have also been reported to produce factors that promote chemoresistance in ovarian cancer cells.[10] HPMC also produce dipeptidyl peptidase IV and VEGF in response to malignant ascites exposure.[13,14]

In addition to the complex mixture of stromal cellular components, malignant tumor cells are found in ascites and are thought to be a major factor in disease recurrence in EOC patients.[15] Tumor cells within the ascites are present either as single cells with adherent properties or, more commonly, as aggregates of non-adherent cells, also known as spheroids.[16] These cells are proposed to undergo epithelial-to-mesenchymal-transition to a motile phenotype with low levels of E-cadherin and higher invasivity than the primary tumor cells.[17,18] Spheroids represent the invasive or metastasis-forming subpopulation leading to recurrent disease.[19,20] This notion has in part been supported by *in vitro* work on artificial spheroids. However, a study by Wintzell *et al.*[16] showed that the spheroids freshly isolated from patients were less invasive and expressed low levels of the EOC tumor-initiating cell marker CD44 and the stem cell transcription factor OCT4A. However, these spheroids represent a chemoresistant population since chemotherapeutic drugs do not penetrate such multicellular structures.[21,22]

Interestingly, two distinct types of adherent cells have been isolated from ascites, and were separated into mesenchymal-

like and epithelial-like morphology. Both cell populations resembled stem/progenitor cells with potential for self-renew and expression of typical cancer stem cell markers, including $CD44^{high}$, $CD24^{low}$ and $AC133^{+}$.[23] These cells also highly expressed genes associated with tumorigenesis and metastasis, including BMP-2, BMP4, TGF-β, EGFR and integrin $\alpha_2\beta_1$.[23] Current studies are focusing on elucidating the importance of these cellular components in EOC progression.

Acellular components. Cellular components of ascites communicate with each other through soluble factors, including cytokines, proteins and metabolites, and, as discovered recently, through the secretion and exchange of exosomes.[24] Thus, the heterogeneous mixture of cellular components of ascites influences the acellular components of ascites. The acellular components of ascites constitute a dynamic reservoir of both pro-tumorigenic and anti-tumorigenic factors, including cytokines, growth factors and bioactive lipids, which individually and in combination influence EOC behavior and progression.[25–27]

(1) Cytokines in ascites: The cytokine profiles of EOC ascites demonstrated the presence of both pro-tumorigenic and anti-tumorigenic factors in this unique tumor microenvironment.[1,4,28,29] At significantly elevated levels of pro-tumorigenic cytokines including IL-6, IL-8, IL-10, IL-15, IP-10, MCP-1, MIP-1β and vascular endothelial growth factor (VEGF), and significantly reduced levels of IL-2, IL-5, IL-7 and IL-17, PDGF-BB and RANTES were reported in EOC ascites. These factors cumulatively contribute to creating a pro-inflammatory and immunosuppressive tumor microenvironment.[29] Among these factors, IL-6 and IL-10 have received the most attention due to their correlation with poor prognosis and response to therapy.[4,30]

(2) Metabolites in ascites: The metabolomics of EOC ascites are also of interest to determine the role of metabolites in EOC ascites. Metabolome profiling of ascites have demonstrated that the most important differences are found in fatty acids, cholesterol, ceramide, glycerol-3-phosphate, glucose and glucose-3-phosphate in ascites derived from patients with EOC compared to those from patients with portal alcoholic cirrhosis. 2-Hydroxyisovalerate was found to be the most depleted among the other metabolites, whereas glucose-1-phosphate (G1P) was the dominant metabolite in the malignant ascites. The breakdown of branched chain amino acids produces 2-hydroxyisovalerate in humans[31] and has been identified in the urine of patients with lactic and ketoacidosis;[32] its elevation suggests increased amino acid catabolism. The cause of 2-hydroxyisovalerate depletion in EOC ascites is currently unknown. G1P is a product of glycogenolysis and its elevation suggests an increase of glucose usage in the ascites tumor microenvironment.[33] However, measurements of both glucose uptake and lactate production are needed to confirm whether the difference in the metabolite patterns in the ascites tumor microenvironment reflects metabolic reprogramming in cancer cells. Previously, our group have also reported effective use of positron emission tomography using 18F-fluorodeoxyglucose for the diagnosis of ovarian cancer [34] and have demonstrated potential selective cytotoxicity through targeting distinct metabolic characteristics in ovarian cancer cells.[35] Moreover, glucose transporter (GLUT) 1 or GLUT3 and glycolytic enzymes, hexokinase (HK) II are overexpressed in several tumor cells and were proposed as an indicator of poor prognosis in various malignancies, including ovarian cancer.[36] Our group previously demonstrated that overexpression of HK II was associated with chemoresistance and poorer progression-free survival in patients with ovarian cancer.[37] In addition, glycolate, glucose, furanose and fructose were significantly decreased, whereas glycerol-3-phosphate, cholesterol, ceramide and monoacylglycerol; MAG (18:0/0:0/0:0) were significantly elevated in EOC patient-derived ascites.[38] Furthermore, ceramide, derivatives of fatty acids[39] and LPA were identified only in malignant ascites.[38]

(3) Proteins in ascites: Proteomics studies of EOC ascites have revealed the presence of over 2000 proteins.[38,40] Among these, the concentrations of pyruvate kinase isozymes M1/M2 (PKM1/2), glyceraldehyde phosphate dehydrogenase (GAPDH) and mesothelin (MSLN) were slightly but significantly higher in the ascites from patients with serous-type EOC compared to those in the ascites derived from benign ovarian tumor patients.[41] Moreover, the most pronounced differences (≤7-fold) in protein levels were found for the components associated with RNA splicing in EOC-derived ascites compared to those in cirrhosis ascites.[38]

(4) Exosomes in ascites: Exosomes have also been detected in biofluids, including EOC ascites, which are nano-sized microvesicles (30–100 nm in diameter), formed by inward budding of the late endosomal membrane to segregate the cargos, including lipids, proteins and nucleic acids, within the membrane-covered vesicles.[24] Exosomes contain molecular signatures of donor cells and can circulate throughout the body, potentially transferring information between cells to alter gene expression in recipient cells.[42] Moreover, exosome-derived molecular cargos were found to contain distinct subsets of disease-specific biomarkers, including miR-200c, miR-214, CA125, Muc-1 and CD24.[24,43]

Ascites tumor microenvironment contributing to cancer progression and heterogeneity

Ascites serves as an important tumor microenvironment, enriched with pro-tumorigenic signals that fosters the acquisition of hallmarks in ovarian cancer, including proliferation, invasion and anti-apoptosis and subsequently contribute to chemoresistance and tumor heterogeneity.[7,8]

Promoting cancer cell progression. The essential role of ascites in mediating and sustaining the hallmarks of ovarian cancer are being increasingly recognized, where the reciprocal reprogramming of both cancer cells and components of ascites occurs throughout disease progression. Ascites-derived cellular components, including stromal progenitor cells, mesothelial cells, mesenchymal cells and endothelial cells, have been reported increase tumor growth and metastasis.[9,10,44,45]

In particular, ascites-derived mesothelial cells and endothelial cells secrete soluble factors, including osteoprotegerin (which acts as a pro-tumorigenic factor), promote tumor growth and angiogenesis, and attenuate TRAIL-induced apoptosis.[10,45,46] It has been demonstrated that interactions between tumor cells and mesenchymal stem cells promote the elevation of IL-6, a proinflammatory cytokine. IL-6 acts as an oncogenic stimulus, promoting the epithelial–mesenchymal transition process that enables invasion.[44] VEGF is best known as a key regulatory molecule enabling hallmarks of cancer, including tumor growth, invasion, metastasis and recurrence of EOC.[47] Substantial evidence also supports VEGF as a key player in the formation of ascites and ovarian cancer progression.[48,49] Another factor, IL-8, is present in abundance and has been shown to activate the epithelial–mesenchymal transition and metastasis,[50] and enables tumor growth and angiogenesis.[51]

Tumor heterogeneity. Cancers may arise from more than one founder cell, contain subpopulations driven by stochastic, stem

cell and mixed hierarchies, and are exposed to post-transcriptional and environmental influences resulting in their heterogeneity.[52,53] The tumor mass is composed of cancer cells that differ greatly from each other in their morphology, gene expression patterns, metabolism, proliferation and metastatic potential.[54] Heterogeneity is, therefore, both genetic, in which mutations are present on the genome and transferred to subsequent generations, and responsive, whereby the expression of genes is altered by environmental factors or in response to selective pressures.

Epithelial ovarian cancer has long been considered a histologically heterogeneous cancer[1,55,56] as it comprises at least five distinct histological subtypes, the most common and well-studied being high-grade serous ovarian cancer (HGSOC).[55] Pathological and epidemiological studies suggest distinct tissues of origin for the main EOC histotypes. For example, the low-grade endometrioid and clear cell histotypes of EOC are thought to be derived from endometriotic tissue that has migrated along the fallopian tube onto the ovary[57] and mucinous EOC from Walthard nests, which is derived from benign clusters of epithelial cells with morphological similarities to urothelial tissue present at tubal–mesothelial junctions.[58] The strongest association has been made between HGSOC cancers and fallopian tube premalignant lesions.[59] There was considerable genetic heterogeneity observed between patients and between samples collected from the same patient, manifested as chromosome deletion, microsatellite instability and single nucleotide polymorphism (SNP) variation.[60] It is now evident that tumor cells within the ascites are also heterogeneous at both cellular and molecular levels,[55] but its contribution to the tumor heterogeneity and ovarian cancer prognosis need to be studied further. Cancer was previously viewed as a heterogeneous disease, caused not only by tumor cell themselves containing aberrant mutations but also by microenvironment constituents.[61] Chronic and often uncontrolled oncogenic signals are generated from growing tumors and concentrate in ascites, and the components of ascites continually change during disease progression. The proportion and diversity of bioactive molecules present in ascites vary according to the disease subtype, stage and grade, as well as between patients, thus diversifying the constituents of the tumor microenvironment. The heterogeneity in ascites is demonstrated by the presence of both oncogenic and tumor suppressive factors. Specifically, ascites in high-grade serous ovarian cancer patients has been shown to serve as a protective tumor microenvironment against drug-induced apoptosis through induction of survival signaling pathways such as PI3K/Akt in tumor cells.[62,63] The extent to which ascites serves as a protective tumor microenvironment against TRAIL-induced apoptosis and chemotherapy is variable.[64] Therefore, further research is needed to identify key players responsible for the tumoral heterogeneity of the tumor microenvironment.

Therapeutic implications of targeting tumor microenvironment

Ascites is highly attractive as a resource for biomarker discovery studies. As opposed to serum, ascites, being a proximal fluid, might reveal events in the early stage of ovarian cancer. Moreover, the concentration of cancer-associated soluble factors is usually much higher in ascites than in serum.[29] Thus, malignant ascites could be a promising biospecimen for investigating diagnostic, therapeutic, as well as prognostic markers.

Utility of ascites as a diagnostic factor. The presence of ascites is not limited to malignant ovarian cancer: ascites is often present in patients with benign ovarian epithelial tumors. The clinical management of ascites associated with malignant tumors is quite different from those associated with benign lesions. However, it remains difficult in the clinic to differentiate benign and malignant ascites, particularly in the early diagnosis of malignant ascites.[65] Currently, cytological detection of ascites has become a gold standard for confirming malignant ascites.[66] This detection shows high specificity, but its sensitivity is low, which can easily result in misdiagnoses and repeated tests after multiple ascites collection, leading to delays in providing optimal therapeutic options and increased discomfort from abdominocentesis.[65] Our group previously suggested that a smaller amount of ascites may correlate with a good prognosis for patients with ovarian cancer.[67] Moreover, its components may also reveal potential diagnostic and prognostic factors. A number of soluble factors are present in abundance in ovarian cancer patient-derived ascites, but few have been validated for their biomarker potential; these are shown in Table 1.

Several laboratory indexes have been reported, including VEGF, matrix metalloproteinase, DNA heteroploid and human leukocyte antigen system-G indexes, which have some value in diagnosing malignant ascites; however, their applications are limited under clinical settings because of the complicated inspection techniques involved.[68–71] These reported tumor markers have some diagnostic sensitivity and specificity for diagnosing malignant ascites, but the diagnostic value of each index differs greatly in malignant ascites induced by different causes because of the complex etiology of malignant ascites.[72] It is thought that no identified tumor markers show high sensitivity and specificity for prediction of the cause of malignant ascites formation in clinical practice. Combined detection of tumor markers in serum and ascites may improve their diagnostic value. Moreover, a recent study by Zhu *et al.*[65] explored the values of tumor markers in serum and ascites for identifying and diagnosing malignant ascites by

Table 1. Profiles of possible diagnostic and prognostic markers in acellular fraction of ovarian cancer patient derived ascites

Family/type	Targeting molecules	Function	Biomarker [otential	References
Cytokine	IL-6	Pro-inflammatory; upregulate VEGF	Ascites formation; chemoresistance; metastasis; survival	4, 29, 30, 43
Chemokine	IL-8	Pro-inflammatory; angiogenesis	Chemoresistance; metastasis; survival	4, 29, 49, 50
Cytokine	IL-10	Immune suppressive	Tumor stage, grade and histological type	29, 30
TNF receptor superfamily	OPG	Bone remodeling; mucosal immunity	Tumor stage; TRAIL-induced apoptosis resistance	10, 44, 45
Chemokine	VEGF	Angiogenesis; cellular growth	Ascites formation; malignant versus benign ascites discrimination; metastasis; survival	29, 46, 47, 48

analyzing the clinical data of patients diagnosed with ascites; their findings suggested that compared to a single index, combined detection of tumor markers in the serum and ascites will significantly improve diagnostic sensitivity and specificity.[65] However, tumor markers that accurately identify malignant ovarian tumors are required for optimal patient management.

Personalized therapy. Personalized therapies have been added to the treatment strategy of malignant ascites. Several lines of targeted drugs have improved progression-free survival in some patients with ovarian cancer. The presence of ascites in women with advanced ovarian cancer may predict the population of women more likely to derive a long-term benefit from bevacizumab, an anti-angiogenic therapy.[73] Intra-peritoneal infusion of catumaxomab, a bispecific monoclonal antibody (anti-EpCAM and anti-CD3) activates immune cells, despite the prevailing immunosuppressive environment of malignant ascites.[74] The relative excess of CD8+ T cells in ascites is reportedly associated with significantly improved overall survival of ovarian cancer patients.[75] Moreover, integrated analysis of ascites at molecular level may provide a powerful platform for discovering indicators of pathological processes or pharmacologic responses to therapeutic interventions, leading to the development of precision medicine.[76,77] These reports highlight the potential use of ascites constituents in diagnostic and prognostic marker screening in ovarian cancer, and an increased understanding of ascites will enable the development of precision medicine.

Conclusion and future perspectives

Ascites, which is a readily accessible source of primary cancer cells and cancer-associated secreted bioactive factors, are underutilized. Although the presence of ascites correlates with a poor prognosis in ovarian cancer patients, its association with chemoresistance is poorly understood. Further studies are needed to highlight both genetic and responsive heterogeneity and to identify chemoresistance mechanisms in ovarian cancer. The contents of ascites may reflect the molecular signatures of the underlying disease, and ascites potentially harbor both diagnostic and prognostic factors, which can be used in biomarker discovery studies. The current stage of the ascites research field provides a foundation for future experiments examining larger numbers of patient samples to validate suggested markers. A more detailed understanding of the relative contribution of ascites-regulated molecules on subsets of ovarian cancer cells will increase the understanding of ovarian cancer biology and will result in improved treatment for patients. In the present study we have summarized the functional roles of ascites in the progression of EOC and have provided a new perspective regarding tumor heterogeneity.

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Disclosure Statement

The authors have no conflict of interest to declare.

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