Journal of BUON **17:** 627-636, 2012

REVIEW ARTICLE

Current approach to epithelial ovarian cancer based on the concept of cancer stem cells

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Summary

Epithelial ovarian cancer (EOC) is the most common ovarian malignancy. EOCs comprise a diverse group of neoplasms, exhibiting a wide range of morphological characteristics, genetic alterations, and biological behaviors. Currently, there is no effective screening for early detection of EOCs and more than two-thirds of EOC patients are diagnosed with advanced stage disease. The major limiting factors in the treatment of EOC patients are recurrence and chemoresistance. Recent studies suggest that EOCs, like other solid tumors, contain distinct populations of cells that are responsible for tumor initiation, maintenance and growth. These cells, termed cancer stem cells (CSCs), display some of the features of normal stem cells and are thought to evade current chemotherapeutic strategies for the treatment of EOCs. Distinguishing CSC-associated antigen profiles may elucidate novel, more sensitive biomarkers for early detection of EOCs and provide molecular targets for the development of new treatment modalities. This review summarizes the current approaches to EOCs based on the concept of CSCs and evaluates their clinical relevance.

Key words: cancer stem cell, chemoresistance, epithelial ovarian cancer, therapy

Introduction

Worldwide, ovarian cancer is the second most lethal gynecological malignancy [1]. Nonspecific symptoms, lack of reliable biomarkers, frequent diagnosis of advanced-stage disease, and the presence of drug-resistant histological subtypes limit the cure rates and prognosis for ovarian cancer patients.

At present, there is no effective screening for early detection of EOCs and more than two-thirds of EOC patients are diagnosed with the International Federation of Gynecology and Obstetrics (FIGO) stage III or IV disease [2,3]. Current treatment of patients with EOC includes surgical resection (debulking), followed by chemotherapy, usually involving platin compounds and a taxane. Despite advances in therapy, recurrence and chemotherapy resistance are still significant clinical problems. In fact, the majority of EOC patients who achieve a complete remission with chemotherapy will ultimately develop recurrent disease. These clinical settings support the hypothesis that EOCs contain a subpopulation of cells, termed CSCs or tumor-initiating cells, which escape therapeutic procedures and have the capacity to sustain tumor progression [4]. Ineffective targeting of CSC populations is responsible for the therapeutic failures and tumor recurrences [4,5]. Efforts to identify specific genetic and signaling pathway alterations in CSCs have led to the discovery of novel biologic targets that can be used to design adjuvant therapies that could potentially overcome chemoresistance and lead to improved response rates and overall survival [5,6].

The last decade has witnessed a great interest in CSCs. According to a consensus definition [7], CSC is a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor. Tumorigenic populations fulfilling the definition of CSCs have been identified in a number of human cancers, such as leuke-

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Received 22-01-2012; Accepted 22-02-2012

mias [8-13], bladder cancer [14], breast cancer [15,16], brain cancers [17], colon cancer [18-20], head and neck cancers [21], pancreatic cancer [22,23], malignant melanoma [24], prostate cancer [25], lung cancer [26], liver cancer [27], Ewing sarcoma [28], and ovarian cancer [29-37] besides others. It is currently not known whether all cancers contain subpopulations of CSCs.

This review summarizes the current approaches to EOC based on the concept of CSCs and evaluates their clinical relevance.

Epithelial ovarian cancer

Ovarian cancer is a heterogeneous disease. EOCs comprise the majority of ovarian cancers and may be classified into 8 distinct histological subtypes, namely serous, endometrioid, mucinous, clear cell, transitional cell, squamous cell, mixed epithelial and undifferentiated [38]. There are major differences in incidence, tumor behavior (low vs. high malignant potential), and clinical outcome between each histological subtype. It has been estimated that ~50% of malignant ovarian tumors are serous carcinomas, while ~25% are endometrioid carcinomas, ~10% are mucinous carcinomas, and ~5% are clear cell carcinomas [39]. However, a study by Seidman et al. [40] reported incidences of 70, 7, 10, and <3% for serous, endometrioid, clear cell, and mucinous carcinomas, respectively, suggesting that traditional distribution figures may vary considerably. In terms of behavior, serous carcinomas tend to be aggressive, high-grade neoplasms that spread rapidly throughout the pelvis, while endometrioid and mucinous carcinomas are usually low-grade tumors, confined to the ovary [41,42]. Clear cell and endometrioid carcinomas, unlike other subtypes, are strongly linked to endometriosis, leading some authors to believe that it may be a precursor to these carcinomas [43-52].

The specific site of origin of EOC is unclear. The most accepted hypothesis was that EOCs are derived from the ovarian surface epithelium and/or cortical inclusion cysts [53,54]. More recently, it has been proposed that some cases of EOC may actually originate from the epithelial lining of the distal fallopian tube, suggesting that if ovarian cancer is a stem cell based disease, the cells of origin are not limited to the ovary [4,55-58].

The "dualistic model" of EOC pathogenesis was recently proposed in an attempt to integrate a growing clinical, pathological, and molecular genetic evidence that supports at least 2 broad categories of EOC designated type I and type II [42,57-60]. According to this model, type I tumors include low-grade serous, low-grade endometrioid, mucinous, clear cell, and transitional cell (Brenner) carcinomas [57]. These tumors generally behave in an indolent fashion, are confined to the ovary at the time of presentation and develop from well-established precursor lesions that are termed "borderline" tumors [57-59]. They lack mutations of TP53 and are relatively genetically stable but each histologic type exhibits a distinctive molecular genetic profile [57]. The most common genetic alterations seen among type I tumors are KRAS and BRAF mutations, both of which activate the oncogenic MAPK signaling pathway [54,57-59,61]. PTEN mutations, which typically result in constitutive PI3K signaling, occur in ~20% of type I endometrioid neoplasms [62]. The MAPK and PI3K pathways are related and they eventually converge upon a common downstream translation factor, eIF4B [63], which may represent an important signaling axis in type I tumor development. WNT and TGF-β signaling pathways are also of potential importance for type I tumor pathogenesis, based on the presence of β-catenin mutations in 16-54% of endometrioid tumors and TGF-β RII mutations in 66% of type I clear cell tumors [42,54]. Interestingly, all of the genes altered in type I ovarian tumors are components of pathways that become intimately related during the process of epithelial-to-mesenchymal transition [64,65]. Type II tumors include high-grade serous and high-grade endometrioid carcinomas, malignant mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas [57]. These tumors are rapidly growing, highly aggressive neoplasms that lack well defined precursor lesions [59]. Type II tumors display TP53 mutations and may exhibit overexpression of HER2/neu and AKT2 [42,54,57-60,66-69].

Cancer stem cell concept

It is well established that tumors are composed of phenotypically and functionally heterogeneous cells. Such heterogeneity has led investigators to renew their interest in an old hypothesis that tumors, like certain normal tissues, are arranged in a hierarchical order in which only certain populations of cells are responsible for generating the multiple cell types within the tumor [4]. CSC hypothesis postulates that tumors contain phenotypically distinct populations of stem-like cells with self-renewal capacity and the potential to reconstitute the entire cellular heterogeneity of a tumor [70].

According to a consensus definition [7], CSC is a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor. CSCs sit at the apex of a hierarchically organized tumor cell populations and are solely capable of dividing asymmetrically to generate an exact copy of themselves (self-renewal capacity) and a

more differentiated progenitor cell [4,71]. These more differentiated progenitor cells divide rapidly to generate large numbers of daughter cells that will form the bulk of the tumor. Moreover, CSCs are thought to be responsible for tumor initiation, progression and metastasis [72].

There appears to be several sources from which CSCs can arise. They may arise from normal adult stem cells, from more restricted progenitor cells, or even from differentiated cells [71,73]. Normal stem cells are the likely targets of mutagenesis, leading to the formation of CSCs as they already possess active self-renewal pathways, whereas induction of self-renewal genes is required to transform differentiated cells [73].

In addition to unlimited self-renewal and proliferative capacities, CSCs are also long-lived and relatively quiescent, allowing them to escape the cytotoxic effects of chemotherapeutic agents that target actively dividing cells. Although the precise mechanisms responsible for chemoresistance are poorly understood, they probably include increased expression of ATP-binding cassette (ABC) transporter proteins and/or detoxifying enzymes (e.g. aldehyde dehydrogenase, reactive oxygen species [ROC] antioxidants) given the potential for increased exposure to toxins throughout the extended CSC life cycle, as well as the disruption of apoptotic pathway mechanisms [24,74-80]. CSCs have also been shown to be refractory to the effects of radiation [78]. It is hypothesized that CSCs possess DNA protective mechanisms that prevent the effects of radiation [78,81].

Cancer stem cells in epithelial ovarian cancer

Cells express a variety of markers on their surface. The expression of these markers has been used to isolate subpopulations of cancer cells for examination of CSC properties.

The first study on the isolation and identification of epithelial ovarian CSCs was reported a few years ago. Bapat et al. [29] identified clonogenic cells isolated from the ascitic fluid of a single patient with advanced serous ovarian adenocarcinoma. These clones, propagated as multilayered spheroids in serum-containing media, possessed stem-like properties and expressed several markers of pluripotency. Also, these clones generated differentiated progeny *in vitro*, formed xenograft tumors *in vivo* and could be serially transplanted in nude mice [29].

Side population cells

Side population (SP) cells are cells with the property of active expulsion of certain molecules (e.g. the vital dye Hoechst 33342), through plasma membrane ABC transporters such as ABC, G2 subfamily/ breast cancer-resistance protein-1 (ABCG2/BCRP1) [4,36,82]. Therefore, SP cells can be efficiently isolated by flow cytometry sorting. SP phenotype, coupled with the expression of stem cell markers, is the hallmark of normal stem cells as well as CSCs [5,83-85]. Increased expression of ABCG2/BCRP1 in SP cells is responsible for chemoresistance [4,5,36,86]. SP cells have been found in several tissues and cell lines, including skin, lung, mammary epithelium, and embryonic stem cells [87-90]. They have also been isolated from malignant tumors, including leukaemia [91,92] and breast [74,93], brain [74], prostate [74], retinoblastoma [94], lung [95], and ovarian [30,36,96,97] cancers.

Using Hoechst 33342 dye efflux, Szotek et al. [30] identified and characterized SP cells from two distinct genetically engineered mouse ovarian cancer cell lines (MOVCAR7 and 4306) with the capacity for self-renewal and production of heterologous non-SP progeny. In *in vivo* assays, SP cells showed a higher tumor-forming ability than non-SP cells. The Müllerian inhibiting substance inhibited the proliferation of both SP and non-SP cells in contrast to the lipophilic chemotherapeutic agents, such as doxorubicin, where SP showed significant chemoresistance. SP cells were also identified in the human ovarian cancer cell lines IGROV-1, SK-OV3, and OVCAR3, and in cells from patient ascites, although in a much smaller number [30]. Also, it was shown that SP cells isolated from ascites derived from EOC patients and from mice inoculated with human ovarian cancer cell lines expressed stem-related cell markers, such as Oct4, Nanog, STELLAR, and ABCG2/BCRP1 [36].

Moserle et al. [96] investigated the presence of SP in EOC and found it in 9 of 27 primary tumor samples analyzed, as well as in 4 of 6 cultures from xenotransplants. In this study, SP cells showed higher proliferation rates, apoptotic resistance, and significant tumorigenic ability compared to non-SP cells. IFN-α, a cytokine that has widely been used to treat solid tumors, exerted significant antiproliferative and proapoptotic effects on primary cultures containing high numbers of SP cells, and was related to a distinctive change in their transcriptional profile, which was not observed when tumors bearing low SP levels were treated [96].

Recently, SP cells were examined using the human ovarian cancer cell line OVCAR-3 [97]. Under optimal processing and staining parameters, only 0.9% of the whole population was sorted as SP cells. The sorted SP cells showed significantly higher colony formation efficiency than the non-SP cells, and only the SP cells could form holoclones. Real-time PCR disclosed that SP cells expressed higher levels of the "stemness" gene Oct3/4 than the non-SP cells did [97].

CD 133

The transmembrane glycoprotein CD133 (prominin-1, PROM1, AC133) was originally identified in hematopoietic stem cells [98,99]. Several investigators have identified CD133 as a potential CSC marker in the solid tumors of brain [17], prostate [25,100], pancreas [101], liver [102], colon [19,20], and more recently in the endometrium [103,104] and ovary [105,106].

Ferrandina et al. [105] were the first to identify CD133 expression in primary human ovarian cancer. In this study, CD133 expression was much higher in primary human ovarian tumors as compared to its expression in normal ovary, benign ovarian tumors and omental metastases. The identified CD133+ cells were almost completely (<1%) non-endothelial in nature, based on the absence of vascular endothelial growth factor receptor 2 (VEGF-R2), endoglin (CD105) and VE-cadherin. Moreover, $CD133^+$ ovarian tumor cells possessed increased clonogenic and proliferative capacities compared to their $CD133^-$ counterparts. However, this study did not identify any relationship between CD133 expression and clinicopathologic features of the disease. Furthermore, the same investigators reported that CD133 expression did not correlate with increased time to progression of disease or decreased overall survival in 160 primary ovarian cancer patients [106].

A few years ago, Baba et al. [107] showed that $CD133^+$ cells derived from ovarian cancer cell lines divide asymmetrically *in vitro*, generating both $CD133^+$ and $CD133^-$ progeny. Moreover, $CD133^+$ cells exhibited increased resistance to platinum-based therapy and were more tumorigenic *in vivo* then their $CD133^-$ counterparts. The same group also determined that expression of CD133 was epigenetically regulated through histone modification and promoter methylation. Similarly, Curley et al. [35] reported that tumor-derived sorted $CD133^+$ cell populations have an increased tumorigenic capacity than $CD133^-$ cells, and they are capable of regenerating a heterogeneous tumor that is similar to the original patient-derived tumor.

In *in vitro* model, as well as in *in vivo* model, Kusumbe et al. [34] found that $CD133^+$ cells contribute to the establishment of tumor vasculature that is critical for tumor cell survival during disease progression. Using mouse models, the same investigators demonstrated that these cells are actively recruited by functional CSCs for generating tumor microvessels through neovasculogenesis [34].

CD44

CD44 is a single chain transmembrane glycoprotein that is ubiquitously expressed. Multiple isoforms of CD44 exist due to extensive alternative splicing of the 19 exons comprising the gene that encodes CD44 [4]. Principally, CD44 functions as an adhesion molecule, mediating cell-cell and cell-extracellular matrix interactions by binding to hyaluronan. CD44 can also activate many intracellular signaling pathways and has been implicated in cell proliferation, cell differentiation, cell migration, cell motility, angiogenesis and metastasis [4,108]. CD44 is critical for the maintenance and survival of leukemic CSCs by keeping these cells in contact with their supportive niche cells [109,110]. CD44 has also been used either alone or in combination as a cell surface marker distinguishing putative CSC populations across a variety of tumor types [15,18,21,23] including ovarian cancer [30-35,111].

Several reports investigating the possible use of CD44 as a prognostic marker in ovarian cancer have yielded conflicting results, with CD44 expression linked to both favorable and unfavorable outcomes [112-115]. This discrepancy may be dependent on the CD44 isoform analyzed.

In addition, CD44 could provide a putative therapeutic target for delivery of novel hyaluronan-paclitaxel copolymers aimed at reducing tumor burden in ovarian malignancies [116]. Actually, the therapeutic potential of anti-CD44 agents has been highlighted by experiments in which targeting of CD44 using specific antibodies, antisense, and CD44-soluble proteins significantly reduces the proliferative and malignant capabilities of various cancer subtypes [108].

CD117/c-KIT

The *c-kit* proto-oncogene encodes a type III receptor tyrosine kinase (CD117/c-KIT). The kinase activity of CD117 is stimulated by binding of its ligand stem cell factor (SCF), which results in the activation of multiple transcription factors that control various cellular processes including cell proliferation, cell differentiation, apoptosis and cell adhesion [4].

Zhang et al. [31] reported that dual positive $CD44^+CD117^-$ cells comprised the ovarian CSC population in primary human ovarian tumors. It was shown that rare fractions of spheroids derived from dissociated primary human ovarian tumor cells and maintained under stem cell-selective conditions possessed self-renewal capacity, over-expressed stem cell markers (Bmi-1, stem cell factor, Notch-1, Nanog, nestin, ABCG2, and Oct-4) and were resistant to current chemotherapeutic drugs (cisplatin and paclitaxel) [31]. Moreover, these sphere-forming cells were tumorigenic and could be serially propagated in nude mice *in vivo* generating tumors

histologically similar to their original primary tumors. In this study, expression of CD44 and CD117 was shown to be enriched in these non-adherent spheroids. Prospective fluorescence-activated cell sorting (FACS) and injection assays of primary and spheroid-derived xenograft ovarian tumor cells indicated that rare subpopulations of $CD44^+/CD117^+$ cells comprised a highly tumorigenic population in primary human ovarian cancer [31].

Recently, Kusumbe and Bapat [111] used the vital membrane-labeling dyes PKH67/PKH26 to identify a quiescent cell subpopulation in the A4 cell line established from malignant ascites from a patient with high-grade serous ovarian adenocarcinoma [29], as well as commercial human tumor cell lines NT2, PA1, HL60, C6, U87, and T47D. The authors proposed that EOCs consist of three distinct populations: (1) label-retaining PKH^{hi} cells, suggested to be slow-cycling/quiescent - the candidate EOC stem cells; (2) PKH^{lo} cells that undergo partial label dilution, indicative of limited divisions - the candidate tumor progenitor cells; and (3) PKH^{neg} cells that undergo total dye quenching, suggestive of consecutive, rapid divisions - "differentiated" tumor bulk cells [111]. Metastases-derived cells also showed three presented fractions. PKH^{hi} cells showed CSC characteristics, such as self-renewal, high tumor-forming ability in xenograft assays, and the expression of stem-related markers Oct4, Nestin, Nanog, Bmi, CD44, and c-Kit. The identification of EOC stem cells as label-retaining PKH^{hi} cells that undergo reversible quiescence through functional assay of clonogenicity *in vitro* and tumorigenicity *in vivo* provided the first indication of their involvement in tumor dormancy [111].

However, there have been conflicting reports regarding CD117 expression in ovarian cancer. Although Szotek et al. [30] determined that SP cells derived from the mouse ovarian cancer cell line MOVCAR7 were enriched for c-KIT expression, their parallel analyses of human ovarian cancer cell lines and ascites-derived cells indicated no positive c-KIT expression. In their screening of multiple human ovarian primary and ascites tumor cells and xenografts derived from human ovarian tumors, Curley et al. [35] detected no significant expression of CD117 in any source. The discrepancy in CD117 expression in ovarian tumors may be due to differences in the specific antibodies used or the methods of tumor propagation employed (*in vitro* spheroid culture vs. direct *in vivo* propagation).

MyD88

Myeloid differentiation factor 88 (MyD88) is an intracellular adaptor molecule associated with the Toll-like receptor (TLR) signaling pathway. TLRs play critical roles in the control of infection, tissue renewal and repair and have also been implicated in tumor formation. After stimulation, cell surface TLR recruits interleukin-1 (Il-1) receptor associated kinase via MyD88, thus inducing activation of the nuclear factor kappa B (NFKB) and mitogen activated protein kinase signaling pathways [4,117].

In their study, Alvero et al. [32] identified cells from ascites and solid ovarian tumors which were characterized by $CD44^+$, $MyD88^+$ expression, NFKB activity, cytokine and chemokine production, high capacity for repair, chemoresistance to conventional chemotherapies, resistance to tumor necrosis factor α-mediated apoptosis, capacity to form spheroids in suspension, and ability to recapitulate the original tumor *in vivo*. Ovarian CSCs expressing TLR4 and MYD88 would thereby respond to TLR4 ligands by activating NFKB, suggesting that the TLR4 pathway may play a critical role in the process of aberrant repair/differentiation triggered by the CSCs [82]. Another report [33] from the same research group indicated that $CD44^+$/VE-cadherin$^-$/$CD34^-$ cells in ovarian cancer, which they termed Type I EOC cells, could serve as progenitors for tumor vascularization. This report also indicated that this neovascularization process was I kappa B kinase-beta (IKK-beta) dependent, but independent of VEGF.

Therapeutic implications

One of the greatest clinical challenges and the most important causes of failure in EOC treatment is the development of chemoresistance. A significant number of patients that initially respond to standard combinations of surgery and chemotherapy later develop a recurrent, therapy-resistant lethal disease [2,6]. The CSC hypothesis maintains that even if a small number of CSCs remain after therapy, disease recurrence can occur. In contrast, if CSCs are eliminated, the possibility of recurrent disease is minimal [73].

To date, three principal methods for eradicating CSCs have been proposed: direct targeting of CSCs, induction of CSC differentiation/proliferation, and destruction of the supportive niche/stromal microenvironment [4].

Direct targeting of cancer stem cells

Drugs designed to target CSCs may be effective therapeutic agents. Whereas there is overlap in cell surface marker expression and signaling pathways associated with normal stem cells and CSCs, these drugs must sufficiently discriminate between these populations to prevent off-target effects [4].

In ovarian cancer, treatment of slow-proliferating ovarian cancer cells with 7-hydroxystaurosporine was cytostatic and a similar effect was observed when these cells were grown as spheres under stem-cell selective conditions [118,119]. Inhibition of functional hyaluronan-CD44 interactions in CD133-positive primary human ovarian carcinoma cells by small hyaluronan oligosaccharides reduced the association of drug transporters and receptor tyrosine kinases with CD44 and inhibited tumorigenesis of the treated cells [120].

Also, cyclopamine, a plant-derived steroidal alkaloid and specific hedgehog (Hh) pathway inhibitor, inhibits the growth and proliferation of ovarian cancer cells *in vitro* and tumor formation *in vivo* [121]. More recently, several studies have indicated that Hh signaling may achieve its tumor-promoting effects indirectly through paracrine Hh activation in surrounding stromal cells, resulting in a more favorable environment for tumor growth [122,123]. A multicenter clinical trial has been initiated by Genentech using ovarian cancer patients in second or third round complete remission following chemotherapy evaluating the efficacy of Hh inhibitor GDC-0449 as maintenance therapy to improve progression-free survival [4]. Also, there are a number of other versions of Hh pathway inhibitors in various stages of trials or development that appear promising [4].

Park et al. [124] reported Notch3 gene amplification in 19.5% of high-grade serous ovarian carcinomas and demonstrated that inactivation of Notch3 with a gamma-secretase inhibitor suppressed cell proliferation and induced apoptosis in cell lines that overexpressed Notch3.

Also, Rask et al. [125] reported significant overexpression of several Wnt pathway proteins in ovarian cancer compared to normal ovarian tissue, which suggests that this pathway could provide useful targets for treatment. Ovarian endometrioid carcinomas have been reported to be particularly susceptible to mutations in the beta-catenin gene that lead to constitutive Wnt pathway activation [126-128]. However, to date there is a paucity of information regarding the effects of Wnt inhibitors on ovarian tumor growth. Imatinib mesylate (STI-571/*Gleevec/Glivec*), a Bcr-Abl kinase inhibitor, has been shown to effectively inhibit beta-catenin signaling and suppress cell proliferation of colon cancer cell lines and could provide similar therapeutic effects in ovarian cancer [4,129].

Induction of cancer stem cell differentiation/proliferation

Induction of differentiation and/or proliferation of CSC populations could aid in their eradication. Driving CSCs to differentiate would deplete tumors of the drug-resistant populations. Also, inducing CSC proliferation would make the cells sensitive to destruction by standard chemotherapy.

Destruction of the supportive niche/stromal microenvironment

Destruction of the supportive niche/stromal microenvironment may be potentially useful for therapy. The impact of the microenvironment on both promoting and inhibiting tumor growth has been demonstrated [130,131]. The extracellular environment is necessary for cell growth and intercellular communication, in addition to various growth factors and chemokines that may enhance tumor cell proliferation and invasion. In contrast, the microenvironment may also stimulate production of antiangiogenic proteins and certain matrix metalloproteases that can inhibit tumorigenesis [132]. The Hh signaling pathway may promote tumor growth through paracrine activation of its surrounding stromal microenvironment and thus may provide a putative target pathway [122,123].

Conclusion

Ovarian cancer is a heterogeneous disease with various histological subtypes, and it is highly probable that CSCs are involved in EOC development. Despite the number of studies attempting to isolate ovarian CSCs, no well-characterized ovarian CSC antigen profiles have been established. The development of chemoresistant disease represents a major obstacle to successful treatment of EOC patients, and the identification of a molecular profile of ovarian CSC may aid to the development of more effective targeted therapy. A multitargeted approach aimed at destroying bulk tumor cells, CSCs and their supportive microenvironment may provide the most efficient way to treat EOC patients.

Acknowledgements

This work was supported by the Grant No. 175092, from the Ministry of Science and Technical Development of Serbia.

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