Title – Detecting Ovarian Cancer Using Extracellular Vesicles: Progress and Possibilities

Authors

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Abstract

Ovarian cancer (OC) is the deadliest gynecological malignancy. Most patients are diagnosed when they are already in the later stages of the disease. Earlier detection of OC dramatically improves the overall survival, but this is rarely achieved as there is a lack of clinically implemented biomarkers of early disease. Extracellular vesicles (EVs) are small cell-derived vesicles that have been extensively studied in recent years. They contribute to various aspects of cancer pathology, including tumour growth, angiogenesis and metastasis. EVs are released from all cell types and the macromolecular cargo they carry reflects the content of the cells from which they were derived. Cancer cells release EVs with altered cargo into biofluids, and so they represent an excellent potential source of novel biomarkers for the disease. In this review we describe the latest developments in EVs as potential biomarkers for earlier detection of OC. The field is still relatively young, but a number of studies have shown that EVs and the cargo they carry, including miRNAs and proteins, can be used to detect OC. They could also give insight into the stage of the disease and predict the likely therapeutic outcome. There remain a number of challenges to the use of EVs as biomarkers, but through ongoing research and innovation in this exciting field there is great potential for the development of diagnostic assays in the clinic that could improve patient outcome.

Keywords

Extracellular vesicles, exosomes, ovarian cancer, miRNAs, diagnosis, liquid biopsy.

Introduction

Ovarian cancer (OC) is the 7th most commonly diagnosed cancer in women and the disease causes more than 150,000 deaths around the world each year, making it the deadliest gynecological

malignancy[1, 2]. The 5-year survival for OC patients is less than 50%[3], and this is mostly due to the frequently late presentation of the disease (when the cancer has usually already spread to the peritoneal space) and the subsequent acquisition of therapeutic resistance during treatment[3, 4].

OC is a heterogeneous disease that is thought to originate from the epithelium of the ovaries[5], though recent studies suggest that it may originate from cells shed from the fallopian tube[6]. It can be classified into two groups based on their distinct origin and histological characteristics[5]. The first group, originating from epithelial cells and known as epithelial ovarian cancer (EOC), accounts for almost 90% of all OC cases; this group can be further subdivided on the basis of histological morphology into serous (the majority of these are high-grade serous carcinoma [HGSC] and the rest are low-grade serous carcinoma [LGSC]), mucinous, endometrioid, and clear cell carcinomas[7]. The second group can originate from stromal and germ cells and include non-epithelial ovarian cancers that account for 10% of all OC cases[8].

The treatment of most patients with EOC comprises surgical debulking of the tumour mass followed by chemotherapy with platinum-based compounds such as carboplatin, cisplatin and oxaliplatin[9]. Successful debulking is the best correlate for subsequent survival[10, 11]. The treatment regime for EOC has changed little in the past few decades, with the addition of taxanes such as paclitaxel (which work by inhibition of microtubule function) being the only substantial change to the way in which EOCs are treated[9]. The report that the presence of tumor infiltrating lymphocytes is a good indicator of chemotherapy response and improved survival for OC implies a synergistic effect of immunotherapy and chemotherapy that may benefit some patients[12].Immunotherapy for OC is still limited to clinical trials and only a low proportion of patients appear to benefit clinically, unlike immunotherapy treatment in melanoma which appears to be particularly successful[13]. There is therefore an urgent need for improved therapeutics for OC, but most importantly, an urgent need for the identification of improved high-sensitivity biomarkers to facilitate earlier detection of the disease and to monitor treatment response to standard and new therapy.

Current biomarkers for ovarian cancer detection

Almost 70% of OC cases are diagnosed at an advanced stage (III or IV) of the disease[7,14]. Early symptoms of OC, including back pain and digestive problems such as dyspepsia, are general and non-specific and are often related to benign conditions. It tends to be only later, once the less serious causes of symptoms have been eliminated and/or symptoms become more intense or severe, that the cancer is diagnosed[15]. Late diagnosis has a significant impact on prognosis; patients that are diagnosed in earlier stages have a five-year survival rate of >70% but this drops to <40% for those diagnosed in later stages[4, 16]. Transvaginal ultrasonography is an imaging-based approach, which could be used to identify earlier-stage disease, but it is invasive and trials have shown that as a screening tool it only has limited ability to reduce overall survival[17-19]. The inability to detect OC at an earlier stage reveals one of the biggest challenges in biomarker research: the

need of finding a highly sensitive, non-invasive screening method applicable to an asymptomatic population subject to develop OC during their life.

Due to the high incidence and low survival rates, much research effort has been put into finding potential biomarkers for screening the disease. One of the first OC biomarkers identified was Cancer Antigen 125 (CA125), also known as MUC16[20]. CA125 is a high molecular weight trans-membrane glycoprotein produced by coelomic epithelium, and thus far it is the most clinically utilised biomarker for monitoring the response to treatment and detecting disease recurrence[21]. When the treatment is successful the level of circulating CA125 can decrease, while increased levels are correlated with drug resistance and disease progression[22]. Although CA125 is used in the clinic, it has some limitations for the purpose of early detection, as it can be associated with both false positive and false negative results[21]. Non-epithelial ovarian tumours are not associated with increased levels of this mucin, and the various EOC subtypes produce different levels of CA125 (levels are higher in HGSC compared to mucinous carcinoma, for example) [22-24]. Some studies reported a higher correlation between the FIGO (International Federation of Gynecology and Obstetrics) staging and preoperative CA125 levels, whilst other studies suggest the correlation is less clear-cut[23, 24]. CA125 can also be detected in other physiological or pathological conditions leading to false positives; for example, it can be raised in the presence of other cancers including cervix, breast, colon and lung cancer[25], and it can be altered in inflammatory pathologies[26], during the menstrual cycle and in pregnancy[27, 28]. Moreover, in some cases of EOC the level of CA125 is not raised, and this is particularly true in the early stages of the disease when the presence of a biomarker would be most clinically useful[29]. CA125, therefore, does not represent the ideal biomarker for robust and diagnostic detection of early OC, and this is confirmed by clinical trials that reveal CA125 as a screening tool lead to only modest (if any) improvements in patient survival[17, 18].

Another biomarker that has been investigated is the Human Epididymis protein 4 (HE4)[30]. This is a small protein encoded by the *WFDC2* (WAP Four-Disulfide Core Domain 2) gene and secreted by epithelial cells. HE4 is secreted by pulmonary epithelial cells, appears to be involved in sperm maturation, but is also highly expressed in the serum of OC patients when compared with healthy individuals[30, 31] and it could be used for distinguishing OC from benign disease and to monitor treatment and recurrence[32]. Unfortunately, like CA125, HE4 has limitations as a biomarker; its expression has been associated with other conditions (especially with endometrial and lung cancer and cystic fibrosis) and different factors (including menstrual cycle, hormone treatment, age and smoking) can modify its expression, leading to false negatives and false positives[28, 33]. .

Greater predictive power could be achieved by combining different biomarkers. For example, the Risk for Ovarian Malignancy Algorithm combines CA125 and HE4 levels, leading to increases in both sensitivity and specificity and thus diagnostic accuracy[35]. The Risk of Malignancy Index combines CA125 levels, ultrasound results and menopausal status[36]. Another multivariate index assay known as OVA1 combines the results of measuring several different proteins alongside

CA125[37]. However, even these multiparametric tests suffer from false positives and negatives and the only definitive way to diagnose patients is during surgery, making these tests unsuitable for routinely screening OC in the female population. Further research is therefore needed to identify other potential biomarkers for earlier detection of OC.

Extracellular Vesicles as cancer biomarkers

Extracellular vesicles (EVs) are a heterogeneous group of cell-derived submicron vesicles surrounded by a lipid bilayer[38]. EVs can be broadly classified into three main types depending on the mechanisms of their biogenesis (Fig 1). Apoptotic bodies are considered to be larger EVs (>1000 nm in diameter, though smaller apoptotic vesicles can also be released) produced by apoptotic cells. Microvesicles (MVs) are a class of EVs produced by outward membrane budding of a cell with sizes ranging between 50 nm and 1 μm. Exosomes are small (30-150 nm in diameter) EVs produced when multivesicular bodies (MVBs) fuse with the plasma membrane leading to the release of the intraluminal vesicles (which, upon release, are then redefined as exosomes). Another class of EVs that is gaining attention in the tumour microenvironment is large oncosomes (LOs). LOs are a big class of EVs (3-4 um diameter), mostly originated from highly aggressive tumor cells and characterized by carrying a variety of oncogenic signals[39, 40]. More recently, the group of David Lyden (Weill Cornell Medicine, New York, US) reported the identification of a new class of EVs, dubbed exomeres[41]. These particles, whose origin and mechanism of formation are still unknown, were found using asymmetric flow field-flow fractionation, are smaller than exosomes (<50nm) and were described as the most predominant particle secreted by cancer cells[41].

EVs were, until recently, thought to primarily perform the role of facilitating the release of unwanted cellular material[42]. While this may be one of their roles, we now know that they can serve a variety of functions[43]. They play significant roles in many biological processes, including angiogenesis and the immune response[43]. EVs are released from all cell types and they carry various types of cargo such as long nucleic acids (including mRNAs, lncRNAs and DNA), short nucleic acids (including miRNAs, vault RNAs, tRNAs and YRNAs), proteins, glycoproteins, carbohydrates, metabolites and lipids[44, 45]. Their biologically active cargo can be transferred into and used by recipient cells, leading to changes in the function of these cells[46, 47]. EVs are therefore an important part of the signaling dialogue that occurs between cells. Many of the EVs produced by cells can make their way into biofluids, including saliva, urine, cerebrospinal fluid, blood, semen, sweat and tears[43]. The molecular composition of EVs partially reflects the molecular landscape of the parental cell; given that this landscape can be altered in cancer, and that EVs can reach biofluids, these vesicles could serve as an easily accessible window into the state of a tumour[48]. Indeed, the very presence in biofluids of vesicles carrying cancer-related cargo could be a diagnostic indicator that a tumour is present.

One approach that is often taken is to identify potential vesicular biomarkers in cancer patients is to initially use cultured cancer cell lines as a proxy. The cargo (miRNAs in particular) of EVs

released by cancer cell lines (compared to non-cancer cell lines) can be profiled using relatively unbiased techniques (such as microarrays or RNA-seq); transcripts that are identified as altered in cancer cell-derived EVs can then be measured in biofluids to see if they are also de-regulated in patients[48]. However, one potential issue is that, even though the foetal bovine serum that the cultured cells grow in has been pre-cleared of vesicles, it still contains bovine-derived EVs carrying bovine miRNAs[49]. Directly testing biofluids using unbiased techniques may, therefore, be preferable as a method for identifying suitable EV biomarkers; however, working with EVs presents several technical challenges and there are a number of pre-analytical variables that affect the interpretation of the results of such experiments[50, 51]. Further work is also needed to establish the most reliable EV isolation strategy for the analysis of vesicular cargo in biofluids[52].

Despite these barriers, there is great excitement for the potential use of EVs as biomarkers to inform clinicians not just on the *presence* of tumours but also on the *state* of the tumour and its microenvironment[48, 53]. EVs can play an active role in the pathology of cancer, contributing to an increase in various undesirable phenotypes such as metastasis[54], angiogenesis[55] and drug resistance[56]. EVs released by stressed cells (including stress induced by the chemotherapeutic agent cisplatin) are able to induce a range of effects in neighbouring tumour cells, including increased invasion, bystander DNA damage and an adaptive response[57-59]. miRNAs are short non-coding RNAs that can repress the expression of multiple genes, meaning that changes in their levels can lead to substantial phenotypic effects in a cell[60]. They are known to be involved in stress response[61] and in mediating drug resistance in ovarian cancer[62-64]. miRNAs can also contribute to other cancer phenotypes, including migration and angiogenesis[65]. For these reasons, the miRNA content of EVs is of particular interest as a potential diagnostic in cancer[48]. A growing body of literature describes the early attempts to capitalize on EV cargo as a biomarker in a variety of cancer types. In the following section we will review current work investigating EVs as biomarkers in OC (Table 1).

EVs as biomarkers in ovarian cancer

Early forays into EV biomarker research in OC focused on simply counting the number of vesicles in circulation. Cancer cells produce more EVs when compared with normal cells, and this could be related to specific conditions in the tumour microenvironment[66]. The levels of circulating EVs were seen to be elevated in patients with EOC and these may correlate with disease stage[67, 68].

For the reasons described in the previous section , the detection of circulating vesicular RNA, particularly miRNA, could be used as a biomarker for OC. In addition, the RNA is protected from degradation whilst encapsulated in vesicles, and sensitive techniques can be used to amplify specific targets from relatively few copies of the nucleic acid, allowing a global view of the complex RNA landscape in these organelles[69, 70]. Many studies have analysed changes of miRNA levels in biofluids such as plasma or serum, but it is not always clear whether these changes represent free circulating miRNAs or those encapsulated in EVs; a more comprehensive review of

non-vesicular biomarkers in OC has been previously published[71]. Here we will focus on studies where EVs have been specifically investigated.

In one early study it was shown that certain miRNAs (including miR-21, miR-141, miR-200a/b/c and miR-214) were more highly expressed in circulating EVs from OC compared to patients with benign disease[68]. In another study the level of miR-200a/b/c and miR-373 were shown to be elevated in circulating EVs of EOC patients[67]. Correlations with stage and lymph node involvement were observed for miR-200a and miR-373, while lower overall survival correlated with levels of miR-200b/c[67]. EV miRNAs were measured in peritoneal or pleural effusions and the level of a combination of miRNAs was correlated with stage, progression free survival and overall survival[72]. An expression signature of eight miRNAs circulating in serum was also shown to distinguish healthy control from patients with OC[73]. Most of these miRNAs were shown to be vesicular when released by cell lines and xenografts[73]. In another study the levels of miR-21 were elevated in ovarian carcinoma EVs[74]. Pan *et al* showed that the levels of a number of miRNAs are altered in the plasma EVs of ovarian cancer patients. Interestingly, the levels of miR-200b correlated with CA125 and overall survival[75]. Urine could also be used as a source of diagnostic EVs. In one study the levels of miR-30a-5p were elevated in the urine of ovarian cancer patients, and this miRNA was shown to be found in EVs[76].

Proteins in EVs have also been investigated as potential OC biomarkers. Interestingly, CA125 has been identified in EVs and its vesicular levels were higher than freely circulating CA125 plasma levels at an earlier stage, suggesting that studying CA125 vesicular levels instead of freely circulating serum CA125 could be used to detect OC earlier[73] .Another study demonstrated that the expression of Claudin 4 protein in EVs obtained from plasma of OC patients was positively correlated with tumor stage, with a sensitivity of 51% and specificity of 98%[78]. Furthermore, the dual measurement of CA125 and Claudin4 inside EVs could be used as a new combination biomarker, although further validation experiments need to be performed[78]. Patients with stage I EOC had a low level of circulating CA125 but high levels of Claudin 4, suggesting that relative levels of the two could be informative[78]. It has been shown that EVs derived from OC patients' plasma contain increased levels of TGFβ1 and melanoma associated antigen 3 (MAGE3) compared with patients with benign disease, suggesting they could serve as potential biomarkers to distinguish between malignant and benign patients[79]. The levels of EpCAM, ADAM10 and EMMPRIN have also been shown to increase in EOC[68, 80]. Zhang *et al* developed a microfluidic device combined with ELISA to detect EVs; with this device they show that in a small cohort of patients the levels of EpCAM in plasma EVs from OC patients was higher compared to controls[81]. CD24 has been associated with poor prognosis in OC and it is highly enriched in EVs from ascitic fluid[82], with most of the CD24 positive exosomes being secreted by tumour cells[82]. Combined measurement of vesicular EpCAM and CD24 can distinguish between patients that are responsive or non-responsive to therapy[83]. Zhao *et al* developed a microfluidic device named 'ExoSearch Chip' to isolate serum exosomes that contain CA125, EpCAM and CD24. When EVs were isolated using antibodies against CA125 it was noted that patient samples contained a greater amount of EVs compared to healthy controls[84]. Similarly, another microfluidic-based platform was used to show

that the number of EpCAM+ EVs is correlated with disease progression in OC[85]. In another study, soluble E-cadherin was found to be released with EVs into ascitic fluid and the levels were able to distinguish between OC and benign disease[86]. Taken together, these results suggest that vesicular proteins have promising potential as biomarkers and that they could be potentially used as point of care testing (POCT) as quick, cheap and sensitive technique that could help to overcame the challenges related with early diagnosis.

Other EV-associated molecules can also be used as biomarkers. A recent study published by Lea *et al* developed an ELISA assay that can detect and bind picogram-levels of phosphatidylserine (PS)-containing EVs from the plasma of OC patients, and, based on the difference in the number of PS-positive EVs, it can differentiate between malignant and benign disease[87]. The use of single EV-methods that can distinguish between subpopulations of vesicles may also be useful in identifying cancer-specific EVs[88]. In another study, microvesicle-associated tissue factor procoagulant activity was able to distinguish between plasma from OC patient and healthy controls[89].

Future perspective

EVs are not the only potential source of non-invasive circulating biomarkers for detecting and monitoring cancer. In particular, the use of cell-free DNA, circulating tumour DNA and circulating tumour cells have shown both diagnostic and prognostic utility in the diagnosis and monitoring of OC[90, 91].

Although EVs have gained a lot of attention in recent years as a potential biomarker for OC and other cancer types, there is still much work required before their potential can be realised.

There remain many challenges associated with working with EVs[48, 50, 52]. There are several methods for extracting EVs but no universal agreement on which technique is most appropriate for diagnostic purposes. The most commonly used techniques are ultracentrifugation (UC) and size exclusion chromatography (SEC), but both of these approaches lead to the enrichment of EVs with different biofluid impurities including soluble proteins and lipoproteins. Combining methodologies, such as SEC and density cushion centrifugation can help to remove lipoproteins and therefore improve EV purity[52, 92]. Antibody-bound magnetic beads that are specific to EV markers such as CD63 can be used to isolate vesicles and enhance purity[92]. Methods that rely on polyethylene glycol-based precipitation result in much lower EV purity but give higher yields[93]; miRNA/protein biomarkers discovered using such methods cannot be definitively attributed as vesicular (unless further validation is performed) but if the biomarker is reproducibly and robustly altered in OC then it has value. Microfluidic approaches for EV isolation are being developed that can be combined with novel sensor technologies[81, 84, 85]; these approaches may be less useful for biomarker discovery (due to the smaller scale of material that they produce) but could be used effectively to detect differences in specific EV cargo in ovarian cancer patients. Further research is needed to develop isolation and detection technology, and to assess the effect of isolation methodology on EV purity.

Purifying EVs from different biofluids presents various challenges[94], and there are many pre-analytical variables that can affect the measurement of biomarkers such as vesicular miRNA and proteins[51]. These include the method of biofluid collection, time of day, whether the patient has fasted and the presence of other conditions. These factors could affect biomarker discovery and the testing of biomarkers in a clinical setting. More work is therefore required to establish the effect of pre-analytical variables on the detection of EV cargo and robust procedures must be put in place for diagnostic applications.

Another challenge to quantifying EV cargo is choosing the most appropriate 'reference gene'. It is not clear, at present, which proteins or RNAs are most appropriate as a reference for normalisation. The normalisation of expression of RNAs or proteins of interest can therefore be problematic, with outcomes depending on the choice of reference. More studies are needed to identify EV content whose levels are the most stable in different conditions and between different individuals.

EVs in any given biofluid are released from a variety of cells. A tumour may contribute EVs to this heterogeneous population, and the proportion of tumour-derived vesicles will increase as the disease progresses. The ability to detect a smaller number of cancer EVs in this sea of normal EVs depends on several factors, including the nature of extraction methodology, the sensitivity of the detection assay and the 'normal levels' of the EV-cargo being measured. In the ideal test, the EV-cargo being detected would be absent in normal biofluid but present in high levels after the appearance of a tumour. The test could be run on a small amount of biofluid at the point-of-care and be sufficiently sensitive to pick up very small numbers of cancer-derived EVs. As the sensitivity of EV-based detection methods increase it may be possible to move beyond the use of a test to monitor treatment/relapse towards a true diagnostic test for early-stage OC in asymptomatic individuals. Ideally, the collection of this biofluid would be minimally invasive for the patients and part of their routine disease follow-up (such as blood collection, for example). Whilst improvements are being made in all these areas, this hypothetical test does not currently exist. Further work is necessary to identify novel potential biomarkers in EVs and develop the technology required to isolate and detect them. Exciting progress is being made in this area which we hope will allow us to unlock the potential of EVs for earlier diagnosis of ovarian cancer in the near future.

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Figures/Tables

Figure1 – Illustration of biogenesis pathways for EVs. Microvesicles (MVs) are directly released by outward budding of the plasma membrane. The precursors for exosomes are formed inside multivesicular bodies (MVBs) as intraluminal vesicles (ILVs). MVBs can fuse with the lysosomes leading to the degradation of their content or fuse with the PM leading to the release of exosomes into the extracellular space. Exosomes can then be taken up by recipient cells through different pathways leading to the transfer of their cargo and potentially modifying the behavior of recipient cells.

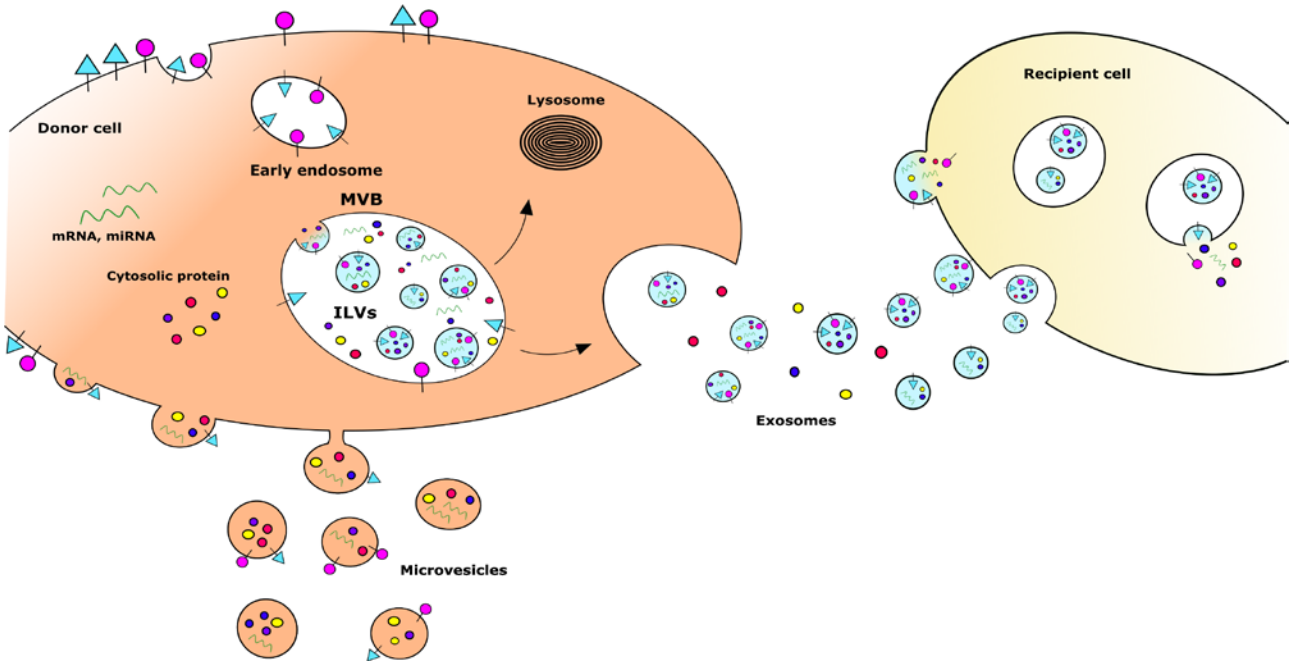

Table1 – EV components identified in EOCs patients that could be used as potential biomarkers for diagnosis or prognosis of ovarian cancer patients.

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Biomarker type	Candidate	Sample source	Comment	Reference
Proteins	CA125	Ascites	Vesicular CA125 higher than circulating CA125 in early stage	Peng P *et al* 2011 [77]
	Claudin 4	Plasma and cell lines	Abundant in EVs from plasma of OC patients in late stages (potential use in combination with CA125)	Li J *et al* 2009 [78]
	TGFβ1 and MAGE3	Plasma	Higher in plasma from OCs patients. Total EV-protein concentration decreased in responsive patients after treatment	Szajnik M *et al* 2013 [79]
	ADAM10, EpCAM and EMMPRIN	Ascites and serum	Ascites EVs correlates with serum EV levels and carry late stage disease markers	Keller S *et al* 2009[80], Taylor D and Gercel-Taylor 2008[68]
	CD24	Ascites and cell lines	High CD24 is associated with poor prognosis in OC and is released in tumour-derived EVs	Runz S *et al* 2007[82]
	CD24 and EpCAM	Ascites	Expression is associated with chemotherapy response	Im H *et al* 2014 [83]
	EpCAM	Serum	EpCAM positive EVs increase in plasma of stage IV patients	Hisey CL *et al* 2018[85]
	E-cadherin	Ascites and non-cancer ovarian cell line	Can help distinguish between healthy and benign disease	Tang MKS *et al* 2018[86]
miRNA	miR-21, miR-141, miR-200, miR-214	Serum	Levels of these miRNAs were similar in the EVs and tumour cells and were predictive of disease stage	Taylor D and Gercel-Taylor 2008[68]

	miR-200 a/b/c and miR-373	Serum	Higher levels in EOC patients. miR200 specific for malignant disease. miR200b/c higher in patients with stage III-IV disease and are associated with CA125 level	Meng X *et al* 2016[67]
	miR- 21, miR- 23b and miR- 29a	Ascites and pleural effusion	Associated with poor survival	Vaksman O *et al* 2014[72]
	miR-142-3p, miR-26a-5p, let-7d-5p, miR-374a-5p, miR-766-3p, miR-200a-3p, miR-328-3p, miR-130b-3p	Serum	Specific for early-stage. Most of these found in EVs	Yokoi A *et al* 2017[73]
	miR-21	Ascites	Up-regulated in malignant cells and tumour-derived EVs	Cappellesso R *et al* 2014[74]
	miR-200b and miR-320	Plasma	EV levels higher in stage IV patients. Both miRNA are higher in patients compared with healthy controls and they are positively correlated with CA125	Pan C *et al* 2018[75]
	miR-30a-5p	Urine	Higher in urine of ovarian cancer patients, particularly in stage I and II	Zhou J *et al* 2015[76]
Other	Phosphatidylserine (PS)	Plasma and cell lines	Higher in cancer-derived EVs	Lea J *et al* 2017[87]
	Microvesicle-associated TF procoagulant activity	Plasma	Improved diagnostic benefit when combined with CA-125 levels	Claussen C *et al* 2016[89]

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