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Review

Extracellular vesicle contents as non-invasive biomarkers in ovarian malignancies

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Ovarian cancer most commonly presents at an advanced stage where survival is approximately 30% compared with >80% if diagnosed and treated before disease spreads. Diagnostic capabilities have progressed from surgical staging via laparotomy to image-guided biopsies and immunohistochemistry staining, along with advances in technology and medicine. Despite improvements in diagnostic capabilities, population-level screening for ovarian cancer is not recommended. Extracellular vesicles (EVs) are 40–150 nm structures formed when the cellular lipid bilayer invaginates. These structures function in cell signaling, immune responses, cancer progression, and establishing the tumor microenvironment. EVs are found in nearly every bodily fluid, including serum, plasma, ascites, urine, and effusion fluid, and contain molecular cargo from their cell of origin. This cargo can be analyzed to yield information about a possible malignancy. In this review we describe how the cargo of EVs has been studied as biomarkers in ovarian cancer. We bring together studies analyzing evidence for various cargos as ovarian cancer biomarkers. Then, we describe the role of EVs in modulation of the tumor microenvironment. This review also summarizes the therapeutic and translational potential of EVs for their optimal utilization as non-invasive biomarkers for novel treatments against cancer.

INTRODUCTION

An estimated 21,410 patients will be diagnosed with ovarian cancer in 2021 and approximately 13,770 will succumb to the disease this year.[1] Nearly 75% of patients with high-grade serous ovarian cancer (HGSC), the most common histology, are diagnosed at advanced stage, where 5-year survival is approximately 32% compared with >80% if diagnosed when disease is localized to the ovary.[2] Thus, detecting ovarian cancer early before distant spread is imperative to improving a patient's chance of survival.

The practice of surgical staging for ovarian cancer was established following Young et al.'s report that 77% of patients with seemingly localized disease (stages IA-IIB) in fact had upper abdominal metastasis.[3] Surgical staging includes the following: aspiration of ascites or pelvic washings; inspection and biopsy of peritoneal surfaces of the pelvis, paracolic gutters, and undersurfaces of the diaphragm; omentectomy; pelvic and para-aortic lymph node dissection; bilateral salpingo-oophorectomy; and hysterectomy when future fertility is not desired.[3,4] Image-guided core biopsy utilizing ultrasound or computed tomography scan has largely replaced surgical biopsy for patients whose performance status, treatment recommendations, or consideration for clinical trial are dependent on histologic subtypes of ovarian cancer or specific genetic variances[5,6] (Figure 1). Both detailed examination of the fallopian tube epithelium in women with a hereditary predisposition to breast and ovarian cancers and IHC staining support a tubal origin of HGSC, as tumors stain positive for Wilms tumor (WT1) and Paired Box 8 (PAX8).[9–12] Nearly all HGSCs harbor *TP53* mutations, resulting in aberrant p53 protein expression, either null-type or diffuse overexpression.[13–15] This limited IHC panel can be done on the smallest of tissue biopsies to establish a diagnosis of HGSC. Following the discovery of a familial link to breast cancer and cloning of breast cancer gene 1 (*BRCA1*)[16,17] and after the 2013 US Supreme Court determination that human genes cannot be patented,[18] *BRCA* mutation testing expanded to multigene panels and direct-to-consumer tests, allowing patients with breast and ovarian cancer and their families to be tested for inherited risks.[19,20] However, these advances in technology, diagnostic, and genomic capabilities did not prevent many ovarian cancer patients from presenting with advanced disease.

In an effort to prevent advanced disease and improve early detection, the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial was undertaken to determine whether screening for ovarian cancer in the general population of the United States could reduce ovarian cancer mortality. After enrolling 78,216 postmenopausal women between 1993 and 2001 there were 118 ovarian cancer deaths in the intervention group and 100 ovarian cancer deaths in the usual care group with median follow-up of 12.4 years (relative risk mortality = 1.18; 95% confidence interval [CI], 0.82–1.71).[21] The PLCO screening study did not reduce ovarian cancer mortality and demonstrated that surgical evaluation performed for abnormal cancer antigen 125 (CA125) or transvaginal ultrasound findings can lead to significant surgical complications.[21] A two-stage strategy for ovarian cancer screening was evaluated by the prospective Risk of Ovarian Cancer Algorithm,[22] which evaluated 4,051 women who underwent

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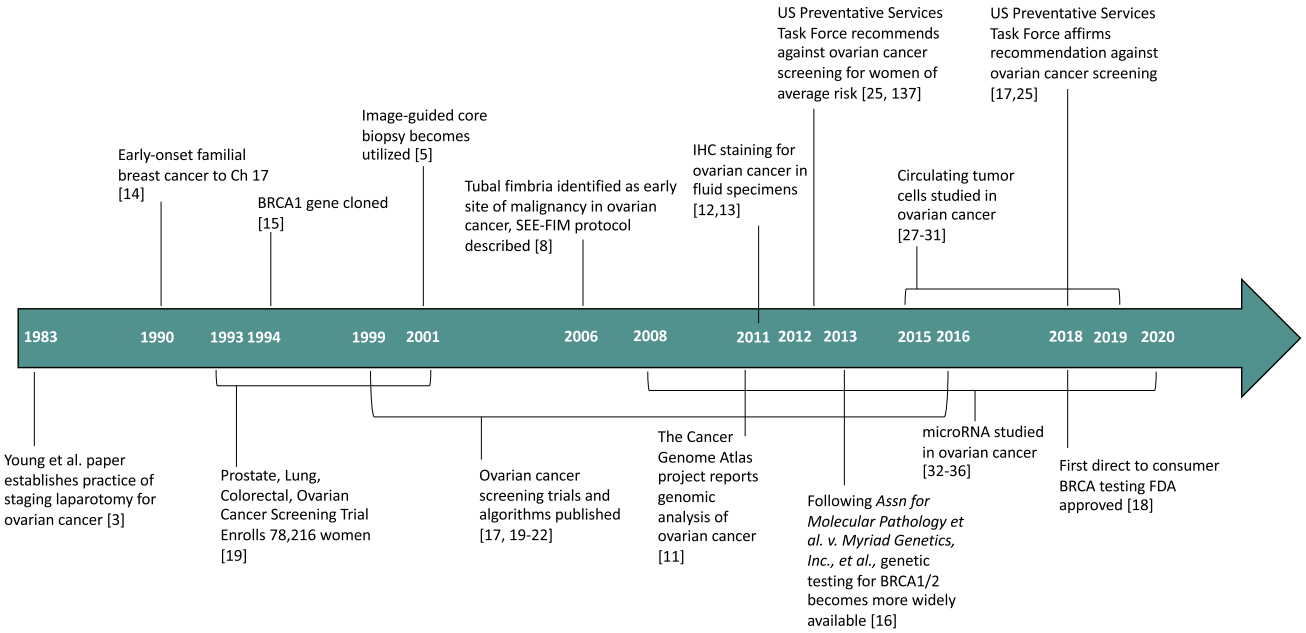

Figure 1. Timeline indicating the development of methods for diagnosis and screening of ovarian cancer[7,8]

annual CA125 testing and were then triaged to three categories based on risk.[22] Ten patients underwent surgery, with four diagnosed with invasive ovarian cancer (1 stage IA, 2 stage IC, 1 stage IIB); two with borderline tumors; one with endometrial cancer; and three with benign ovarian masses (positive predictive value = 40%; 95% CI, 12.2%–73.8%; specificity = 99.9%; 95% CI, 99.7%–100%).[22] A 2018 systematic review assessed four clinical trials of screening interventions, including CA125, transvaginal ultrasound, and CA125 plus transvaginal ultrasound.[21,23–26] After no significant difference in ovarian cancer mortality with screening was found,[26] the United States Preventative Services Task Force (USPSTF) affirmed its 2012 recommendation in 2018 to avoid ovarian cancer screening for women of average risk.[27] As these screening trials and formal recommendations from USPSTF indicate, there is not currently a screening tool or biomarker that can be effectively used for identifying early ovarian cancer.

HGSC is able to both metastasize through the systemic circulation and passively disseminate through the peritoneal cavity, potentially leaving genetic material and metabolites that may be detected through subsequent liquid biopsy.[28] Liquid biopsy is a bodily fluid-based, minimally invasive method that has the potential for early detection of cancer. Circulating tumor cells and circulating tumor DNA appear to be promising in early diagnosis and determining prognosis and therapeutic response.[29–33] Circulating microRNAs (miRNAs) are noncoding RNA fragments that have been detected in nearly all body fluids and regulate expression of target genes within the cell.[34,35] The stability and ubiquity of miRNAs in the circulation, either as cell-free miRNA or within extracellular vesicles (EVs) or exosomes, make them promising tools in detecting ovarian cancer before it has reached an advanced stage.[36–38] Utilizing EV contents as non-invasive biomarkers may help detect ovarian cancer before it has metastasized outside the pelvis. While EV-based biomarkers are not currently used in cooperative group or phase III clinical trials, integral biomarkers are defining who may be enrolled on which ovarian cancer clinical trials, and integrated biomarkers are included in studies testing important hypotheses.[39] The current era of precision medicine brings the study of exploratory biomarkers to the forefront,[39] and with advances in technology, and a need for early detection of ovarian cancer, the study of EVs as biomarkers in ovarian cancer is more relevant than ever. In this review we describe EVs, various cargos found within EVs, and novel technologies to utilize EV contents as biomarkers in ovarian cancer. We then discuss the role of EVs in immune evasion, metastasis, and chemoresistance and impact on the ovarian cancer tumor microenvironment (TME). We conclude with a discussion of EV-based therapeutics and translational aspects of the study of EVs in ovarian cancer.

EVs as biomarkers: The key to non-invasive options

EVs measuring 40–150 nm of endosomal origin are commonly known as exosomes.[40–42] Due to complexity surrounding the nomenclature of EV subtypes, in this review we describe all particles arising from both the endosome and plasma membrane using the generic term, EVs, although specific subtypes, including exosomes originating from the endosome, ectosomes derived from the plasma membrane, microparticles and microvesicles, and EV subtypes based on size (small versus medium/large with size ranges defined) or their biochemical composition exist.[43] EVs are present in all biologic fluids, including plasma, serum, urine, ascites, and effusions.[40] They are formed when the cellular lipid bilayer invaginates, thus containing

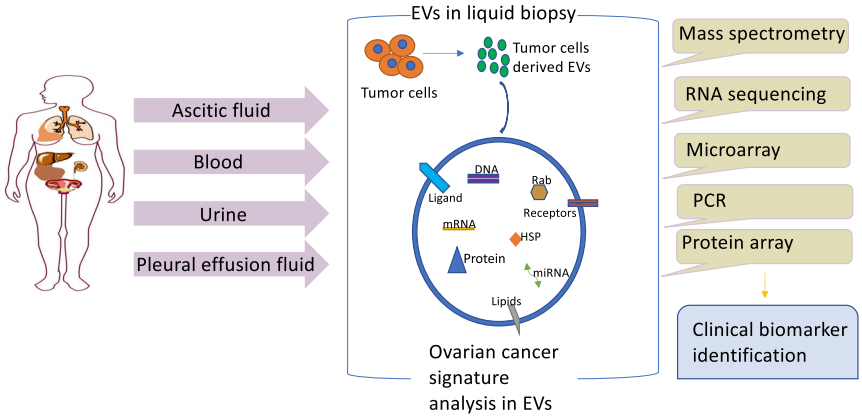

Figure 2. Schema depicts how exosome-carrying agents were employed as a biomarker using non-invasive methods

molecular cargo from the cell of origin, such as DNA, mRNA, noncoding RNA, proteins, lipids, and metabolites.[40] The composition of EVs is determined by the proximity to and the interacting ability of the cytoplasmic components with the budding membrane when the EV is formed.[43] The role of EVs is most dependent on its cell or tissue of origin, and EVs are involved in cell signaling, intercellular communication, immune responses, cancer progression, and establishment of the TME.[40,41,44] The potential uses of EVs are limitless, ranging from early detection of cancers to therapeutic use in drug delivery.[41,44,45]

EVs are commonly characterized by the presence of transmembrane or membrane-anchored proteins, which demonstrate the lipid-bilayer structure specific to EVs and the presence of cytosolic proteins.[43] EVs have the ability to give an insider's view of the environment inside the cell without disrupting the cell.[45] Characterization of EVs in the experimental setting should be completed using several different methods to assess different components of EVs.[43,46] These include but are not limited to the following: western blot of proteins found within EVs or on exosomal membranes, such as TSG101, ALIX, and tetraspanins CD9 and CD63[45,47]; electron microscopy[48,49]; and nanoparticle tracking analyzer,[49,50] among others.

There are several different methods utilized for separating or isolating EVs before further analyses can take place. The first step involves assessing the media, bodily fluid, or tissue from which EVs will be isolated.[43] Although there are specific isolation protocols based on type of culture medium or bodily fluid, broadly these methods may involve forms of centrifugation and ultracentrifugation, filtration, separation using a gradient, polymeric precipitation, microfluidics, or isolation using a commercially available kit.[43,45,51–54] The comparison of ultracentrifugation with four commercially available kits—ExoQuick-TC (System Biosciences), Total Exosome Isolation Reagent for cell culture medium (Invitrogen, Life Technology, Carlsbad, CA, USA), ExoQuick (System Biosciences), and Total Exosome Isolation Reagent for serum (Invitrogen, Life Technology)—was performed by Tang et al.[53] This study revealed a decreased volume of EVs but increased purity when isolated by ultracentrifugation, whereas the commercial kits yielded more exosomes but with more contaminant protein.[53]

The major challenge in exosome research is due to the time-consuming measures and ultracentrifugation processes, which are often required for successful isolation and detection of exosomes from body fluids. Variabilities in exosome isolation techniques and handling of various source samples across researchers may affect the purity, concentration, and size of the EVs when isolating them.[43,53,54] In addition, demographic characteristics of the patients from whom samples are obtained and EVs isolated need to be carefully matched between cases and controls to limit confounding factors.[43,54] The potential impact of these methodological challenges is not to be understated, as reproducibility in the setting of a myriad of isolation techniques is a hurdle. Therefore, detailed and precise documentation of methods in the isolation of EVs is absolutely essential to help overcome this obstacle.[43,54]

Once EVs have been isolated, their contents can be further screened and characterized through RNA sequencing, quantitative PCR array, and protein array (Figure 2), with possible correlations to the clinical care of women with benign and malignant ovarian masses.[41,44,53,55] In contrast to the conventional isolation procedures, advances in nanotechnology and development of microfluidic devices allowed investigators to isolate high-quality exosomes from relatively small quantities of biological fluids.[56,57] Using a different microfluidic chip based on a 3D nanostructured herringbone pattern, Zhang et al. showed that the contents of EVs could be evaluated in 2 μL of plasma. After evaluating plasma from ovarian cancer patients and controls, they determined that EpCAM, CD24, and folate receptor α protein in EVs can be used to discriminate patients from controls.[57] Thus, we may expect improvements in the diagnostic and prognostic utility of EVs with advances in technology and microfluidics.

Use of EVs as biomarkers in ovarian cancer

EVs have been examined in the setting of patients with ovarian cancer as potential diagnostic biomarkers and to monitor responses to therapy. A greater quantity of EVs have been detected in the plasma of patients with ovarian cancer compared with healthy controls.[56,58,59] A common feature of ovarian cancer is the presence of malignant ascites, and malignant ascites contain more EVs than ascites formed due to physiological disorders.[60] In addition, EVs derived from bodily fluid from patients with ovarian cancer contain numerous sources of information, including DNA, mRNA, noncoding RNA including miRNA, and long noncoding

RNA (lncRNA), proteins, lipids, and metabolites.[40,41,55,60] In the sections below we describe how the cargo of EVs has been studied as biomarkers in ovarian cancer (Table 1).

Protein biomarkers in EVs

Szajnik et al. evaluated the protein content of plasma EVs in patients with ovarian cancer, benign ovarian tumors, and healthy controls.[58] They found significantly greater protein content in plasma EVs from ovarian cancer patients (125 μg/mL) compared with benign tumor patients (70 μg/mL) ($p < 0.05$).[56] When examining the protein cargo of EVs, TGF-β and MAGE3/6 were expressed in the plasma EVs of ovarian cancer patients before and during their chemotherapy treatment, whereas there was little expression of TGF-β in patients without ovarian cancer and MAGE3/6 was absent in such patients.[58] These protein markers demonstrate the possible applicability of proteins in plasma EVs to distinguish ovarian cancer patients from benign tumor patients and healthy controls.[58]

In an analysis of EVs from ovarian cancer cell lines and malignant patient ascites, Runz et al. studied the proteins CD24 and EpCAM.[61] CD24 has been associated with decreased overall survival in patients with epithelial ovarian cancer.[94] A strong CD24 level was obtained from EVs secreted by the ovarian cancer cell lines GG and M130.[61] In malignant ovarian tumors, CD24 expression was highly variable, but in EVs isolated from ascites from the same ovarian cancer patients, all samples expressed CD24.[61] EVs isolated from malignant ovarian cancer ascites also contained EpCAM, which is expressed in epithelial tissues and is a marker of carcinoma.[61,95] The presence of both CD24 and EpCAM in EVs from ascites confirm the proteins to be tumor-derived rather than from bone marrow despite the variable CD24 expression in tumor cells.[61]

Li et al. studied claudin proteins in ovarian cancer cell line culture media and demonstrated the presence of claudin 4 (CLDN4) within exosomes through ultracentrifugation, immunogold labeling, and visualization with electron microscopy.[62] They then examined plasma samples of patients with HGSC and controls and through ultracentrifugation and immunoblotting determined that CLDN4 had a sensitivity of 51% and specificity of 98% to detect ovarian cancer.[62] In addition, when using a CA125 cutoff of 125 units/mL as positive, 31/32 patients identified as positive by detection of CLDN4 in plasma exosomes were also positive by CA125 value.[62] Gupta et al. investigated sphingosine kinase-1 (SPHK1) in EVs from different ovarian cancer cell lines (Table 1).[65] In addition, SPHK1 was significantly greater in EVs isolated from plasma of ovarian cancer patients when compared with healthy controls.[65] Mechanistically, EVs containing SPHK1 are transported to the TME where SPHK1 catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P), thus releasing S1P into the TME.[65] S1P then acts as an immunosuppressive agent through T cell exhaustion, which can contribute to ovarian cancer progression.[65] These results suggest the notion that the contents in EVs can be combined with a known biomarker, such as CA125 levels in ovarian cancer, as a predictive marker of response to therapies.

Table 1. List of reported biomarkers and their source in high-grade serous ovarian cancer

Marker	Model and sample	Reference
Protein biomarkers in EVs		
TGF-β, MAGE3/6	human plasma	Szajnik et al.[58]
CD24	*in vitro*: cell lines GG, M130 human malignant ascites	Runz et al.[61]
EpCAM	human malignant ascites	Runz et al.[61]
CD24, EpCAM, folate receptor α	human plasma	Zhang et al.[57]
Claudin 4	*in vitro*: cell line BG1 human plasma	Li et al.[62]
HGF	*in vitro*: cell line OVCAR8	Dorayappan et al.[56]
HGF, STAT3, IL-6	human serum	Dorayappan et al.[56]
EpCAM, PCNA, TUBB3, EGFR, APOE, claudin 3, FASN, ERBB2, L1CAM(CD171)	*in vitro*: cell lines OVCAR3, IGROV1	Liang et al.[63]
TRKB	*in vitro*: cell lines SKOV3, CAOV3, ES2 human serum	Li et al.[64]
SPHK1	*in vitro*: cell lines HeyA8, OVCAR3, OVCAR4, OVCAR5 human plasma	Gupta et al.[65]
LGALS3BP, bisecting-GlcNAc-containing glycans, LacdiNAc	*in vitro*: cell lines OVMz	Gomes et al.[66]
bisecting-GlcNAc-containing glycans	*in vitro*: cell lines SKOV3	Escrevente et al.[67]
MicroRNA biomarkers in EVs		
miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214	human serum	Taylor et al.[59]
miRNA-99a-5p	*in vitro*: cell lines HeyA8, TYK-nu human serum	Yoshimura et al.[68]
miR-373, miR-200a, miR-200b, miR-200c	human serum	Meng et al.[69]
miR-200b, miR-200c	*in vitro*: OVCAR3	Kobayashi et al.[70]
Let-7	*In vitro*: SKOV3	Kobayashi et al.[70]
miR-21	human: malignant ascites	Cappellesso et al.[71]
miR-30a-5p	*in vitro*: SKOV3 human urine	Zhou et al.[72]
miR-101	human serum	Xu et al.[73]
Let-7d-5p	*in vitro*: CAOV3, SKOV3, RMUG-S	Yokoi et al.[74]
miR-142-3p	*in vitro*: ES-2, SKOV3	Yokoi et al.[74]
miR-200a-3p	*in vitro*: RMG-1, OVCAR3	Yokoi et al.[74]

(*Continued on next page*)

Table 1. *Continued*

Marker	Model and sample	Reference
miR-26a-5p	*in vitro*: SKOV3, OV-90	Yokoi et al.[74]
miR-374a-5p	*in vitro*: OV-90, RMUG-S	Yokoi et al.[74]
miR-766-3p	*in vitro*: A2780, SKOV3, RMUG-S	Yokoi et al.[74]
miR-130b-3p	*in vitro*: OV-90	Yokoi et al.[74]
miR-21, miR-100, miR-200b, miR-320	human plasma	Pan et al.[75]
miR-200b	*in vitro*: SKOV3	Pan et al.[75]
miR-320	*in vitro*: SKOV3, OVCAR3	Pan et al.[75]
miR-1290	human serum	Kobayashi et al.[76]
miR-6126	*in vitro*: HeyA8, SKOV3, A2780	Kanlikilicer et al.[77]
miR-1307, miR-375	human serum	Su et al.[78]
miR-21, miR-23b, miR-29a	human malignant effusion	Vaksman et al.[79]
miR-940	*in vitro*: HeyA8, HeyA8-MDR, A2780-CP20, A2780-PAR, SKOV3ip1	Rashed et al.[80]
miR-222-3p	human serum *in vitro*: SKOV3, A2780	Ying et al.[81]
miR-145, miR-200c	human serum	Kim et al.[82]
miR-205	human serum	Wang et al.[83]
miR-200b	human plasma	Xiong et al.[84]
miR-21-5p	human plasma *in vitro*: SKOV3, A2780	Cao et al.[85]
miR-630	*in vitro*: A2780, SKOV3	Cui et al.[86]
miR200c-3p, miR18, miR1246, miR1290	human malignant ascites	Mitra et al.[60]
Long noncoding RNA biomarkers in EVs		
MEG3	human effusion fluid	Filippov-Levy et al.[87]
SPOCD1-AS	*in vitro*: cell lines SKOV3, A2780	Wang et al.[88]
MALAT1	*in vitro*: SKOV3, HO8910 human serum	Qiu et al.[89]
SOX2-OT	human plasma	Lai et al.[90]
mRNA biomarkers in EVs		
NANOG	human effusion fluid	Sherman-Samis et al.[91]
NANOG	human malignant ascites *in vitro*: cell line OVCAR3	Yamamoto et al.[92]
SPINT2	human malignant ascites *in vitro*: cell lines SKOV3, OVCAR3	Yamamoto et al.[92]
DNA biomarkers in EVs		
mtDNA copy number levels	human whole blood and plasma	Keserű et al.[93]

In a recent study, Dorayappan et al. used a novel microfluidic chip to isolate EVs based on exosome surface marker CD63 and epithelial cell marker EpCAM.[56] Using this technology they demonstrated that OVCAR-8 cancer cells release significantly more EVs when compared with EVs originating from fallopian tube secretory cells or ovarian surface epithelial cells.[56] Hepatocyte growth factor (HGF) was upregulated in EVs from OVCAR-8 malignant cells but not EVs from fallopian tube secretory cells or ovarian surface epithelial cells.[56] In addition protein markers HGF, STAT3, and IL-6 were significantly elevated in serum EVs from early-stage HGSC patients compared with serum EVs from benign and stage IV HGSC patients.[56] Taken together, these studies suggest that protein markers in EVs could provide reliable markers that could predict the onset of ovarian cancer.

MicroRNA biomarkers in EVs

MicroRNAs (miRNAs) are single-stranded RNA molecules of approximately 20–25 nucleotides that can regulate gene expression by binding to mRNA leading to translational silencing or mRNA destruction.[96,97] miRNAs can travel through the circulation and are largely protected from RNAse degradation due to their encapsulation in EVs and association with RNA-binding proteins.[97,98] This allows miRNA to serve as potential biomarkers in ovarian cancer.[97]

In 2008 Taylor and Gercel-Taylor analyzed miRNA in EVs from the serum of ovarian cancer patients.[59] Prior to this, ovarian cancer tumor tissue was used as a source of exosomal miRNA,[99] and miRNA in circulating EVs had not been studied in ovarian cancer.[59] Taylor and Gercel-Taylor confirmed previous miRNA aberrations from ovarian cancer tumor samples and found elevated miRNA in 218 of 467 samples tested (46%).[59] When exosomal miRNA were evaluated, 94% (206/218) were the same or elevated in EVs isolated from serum compared with EVs from tumor samples, which suggests that circulating exosomal miRNA can be used as a surrogate of tumor tissue miRNA.[59] In addition, for eight miRNA (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214) the mean miRNA intensity was significantly greater in serum EVs in patients with ovarian cancer compared with benign ovarian disease.[59] In an analysis of exosomal miRNA-99a-5p, expression was significantly increased in the epithelial ovarian cancer cell lines HeyA8 and TYK-nu.[68] MiRNA-99a-5p expression was significantly increased in the serum of both ovarian cancer patients and patients with benign ovarian tumors compared with healthy volunteers (2.8- and 1.7-fold, respectively).[68] Measures of miRNA-99a-5p were then measured before and after primary debulking surgery in 26 patients with epithelial ovarian cancer, and a significant decrease in miRNA expression from 1.8 preoperatively to 1.3 postoperatively was noted ($p = 0.003$), which suggests that miRNA-99a-5p is derived from ovarian cancer cells and reflects the burden of disease.[68]

Meng et al. studied the diagnostic and prognostic significance of concentrations of four miRNA (miR-373, miR-200a, miR-200b, miR-200c) in 163 patients with epithelial ovarian cancer.[69] All four exosomal miRNA could distinguish between healthy controls and ovarian cancer, and three (miR-200a, miR-200b, miR-200c) could separate

benign and malignant ovarian tumors based on serum concentration of exosomal miRNA.[69] Two miRNA (miR-200b, miR-200c) were found to have greater serum concentrations in stage III and IV epithelial ovarian cancer compared with stages I-II, and were associated with serum CA125 levels and shorter overall survival.[69] In a mechanistic study, Cui et al. determined that miR-630 was transported by EVs, and that this miRNA contributes to transformation of normal fibroblasts to cancer-associated fibroblasts (CAFs).[86] Studies from our group also identified that miRNA-569 and miRNA-551b are highly expressed in both ovarian cancer tumors and ascites fluid.[100,101] These results suggest that both miRNA expression and concentration in EVs and circulation of bodily fluids can serve as potential ovarian cancer biomarkers.

lncRNA biomarkers in EVs

lncRNAs are defined as RNAs that are greater than 200 nucleotides in length.[102] While not coding for protein, lncRNAs are involved in gene regulation at transcriptional, translation, and post-translational levels and are thought to participate in the progression of cancers.[102] In an array analysis of 17,696 noncoding RNAs, the majority were expressed in HGSC.[87] Filippov-Levy and colleagues found significant differences in lncRNA microarray results at different anatomic sites, including comparing ovary tissue with metastasis, effusion, and EVs extracted from effusion supernatants, respectively.[87] As the mechanism of action of most lncRNAs is not known, ten lncRNAs with attributed function were then analyzed by qPCR in HGSC solid metastases, ovarian tumors, effusions, and EVs isolated from effusions. Of the ten, five lncRNAs exhibited significantly different expression compared across sites: ESRG (embryonic stem cell related), LINK-A (long intergenic noncoding RNA for kinase activation), MEG3 (maternally expressed gene 3), GATS (stromal antigen 3 opposite strand), and PVT-1.[87] In this study, MEG3 expression was higher in solid tumors and EVs isolated from effusions,[87] and this lncRNA has been studied in breast and lung cancer[103,104] and associated with cisplatin resistance.[104] The differences in lncRNAs between disease sites in high-grade serous ovarian cancer suggests that additional investigation is warranted to elucidate the mechanism by which these lncRNAs may influence the TME or metastatic spread.

Wang et al. treated human mesothelial cells with EVs from ovarian cancer cell lines SKOV3 and A2780 and performed lncRNA sequencing to determine which lncRNAs were altered.[88] One, named anti-sense transcript of SPOCD1 gene (SPOCD1-AS) was upregulated in mesothelial cells treated with ovarian cancer EVs.[88] Further evaluation revealed that SPOCD1-AS had no coding potential, had higher expression in ovarian cancer cells compared with IOSE-80 cells, and was expressed more in cancer EVs than IOSE-80 EVs.[88] In an orthotopic ovarian cancer mouse model, EVs carrying SPOCD1-AS promoted peritoneal metastasis, and when the generation of EVs was inhibited, peritoneal metastasis, but not primary tumor growth, was inhibited, suggesting the role of lncRNA SPOCD1-AS both as biomarker in EVs and contributor to peritoneal metastasis.[88]

mRNA biomarkers in EVs

Sherman-Samis et al. examined EVs isolated from HGOS cancer effusions.[91] They found NANOG mRNA in the exosomes of 89% (71/80) specimens, while the mRNA of other markers, including SOX2, OCT4, and LIN28, was not found in EVs but was identified in effusion cells by immunohistochemistry.[91] There did not appear to be a relationship between NANOG mRNA levels and clinical characteristics of the patients from whom effusion fluid was evaluated.[91] When EVs from NANOG knockout lines were used as a treatment in OVCAR8 and OVCAR3 cells, tumor cells exhibited reduced ability to degrade and invade the basement membrane matrix *in vitro*.[91] In a similar analysis of EVs isolated from serous ovarian cancer ascites and benign peritoneal fluid, mRNA from SPINT2 and NANOG were more highly expressed in malignant ascites compared with benign peritoneal fluid.[92]

DNA biomarkers in EVs

Double-stranded DNA found in EVs represents the parental cell and contains the same mutations found in tumors, such as melanoma.[55] While studying exosomal DNA, Thakur and colleagues noted a greater than 50% reduction in DNA level after dsDNase digestion in melanoma, leukemia, lung, and breast cancer cell lines, which indicates that double-stranded DNA is the predominant form of DNA in EVs.[55]

Keserű et al. investigated mitochondrial DNA (mtDNA) copy number in samples of whole blood and plasma from patients with serous epithelial ovarian cancer.[93] Exosome-encapsulated mtDNA copy number was significantly greater in advanced-stage and all stages compared with controls (stages III/IV, $p = 0.0095$; stages I/III/IV, $p = 0.0155$) but no significant difference was found when comparing exosomal mtDNA between controls and stage I patients ($p = 0.3907$).[93] As demonstrated in this study, mtDNA levels are highly variable, but may provide useful information about disease progression or early-stage ovarian cancer detection.[93]

Novel technologies utilizing EVs for early detection of ovarian cancer

Recent developments in nanoparticle research suggest that spectroscopy combining machine learning algorithms with nanoparticle science can be used to characterize EVs to detect ovarian cancer. Several groups have used surface-enhanced Raman spectroscopy (SERS) to analyze ovarian cancer-derived EVs.[105–108] In a study of serum from patients with ovarian and endometrial cancer, principal-component analysis was able to distinguish each histologic entity from other malignancies and from controls.[105] While this novel analysis shows promise, validation studies, standardization of isolation and processing, and evaluation of both cost and reproducibility are needed.[105] Recently, Ćulum et al.[108] published groundbreaking work in the field of ovarian cancer-derived EVs by investigating multiple ovarian cancer cell lines in addition to SKOV3.[105,108] Their work used principal-component analysis and a machine learning algorithm of logistic regressions involving the cell lines OV-90, OVCAR3, EOC6, EOC18, and hIOSE to differentiate EVs from a normal versus ovarian cancer

cell line, a normal versus high-grade ovarian cancer cell line, a normal versus low-grade ovarian cancer cell line, and a low-grade versus high-grade cancer, respectively.[108] Notably, the SERS method of evaluating ovarian cancer EVs achieved the accuracy, sensitivity, and specificity >98%, which suggests that the SERS method of evaluating EVs holds promise for the early diagnosis of ovarian cancer in the future.[108] In addition to spectroscopy, microfluidic chips continue to advance the technology allowing researchers to investigate the contents of EVs.[56,57] As previously mentioned, Zhang et al.'s work utilizing a 3D nanoparticle chip enabled characterization of contents utilizing only 2 μL of patient plasma.[57]

EVs in ovarian cancer immune evasion, metastasis, and chemoresistance

Immune evasion is the characteristic feature of cancer by which cancer cells can survive by evading immune cells, such as cytotoxic T cells and NK cells. Both tumor-associated EVs and the EVs derived from tumor-associated immune cells may cooperate for tumor progression. For example, the expression of CD47 on tumor-associated EVs appears to be associated with immune evasion and is implicated in reducing the phagocytosis of macrophages in the TME.[109] In addition, tumor-associated macrophages releasing microRNA-29a-3p-containing EVs are found to upregulate the PD-L1 to facilitate ovarian cancer cell proliferation and immune escape.[110]

Tumor cells are known to cause T cell suppression, but the mechanism describing EV-based T cell suppression has only been explored recently. This can be exemplified by the upregulation of Siglec-10 on T cells by EVs derived from malignant ascites.[111] Siglec-10 is the inhibitory receptor expressed on immune cells, which blocks the activity of immune cells.[111] Ovarian tumor-associated EVs have also been found to increase the expression of PD-1, which causes exhaustion whereby T cells become incapable of killing tumor cells.[65] We and others have reported that tumor-associated EVs have been shown to carry different enzymes, including SPHK1 and Arginase-1, which in turn cause immune suppression in the TME by inhibiting T cell activity.[65,112,113] Therefore, further characterization of the contents of EVs, such as enzymes, kinases, receptors, and other cargos, is a promising area which may identify novel immune suppressive mechanisms elicited through both cancer cell- and tumor-associated immune cell-derived EVs.

In their role as cargo deliverers, EVs contribute to ovarian cancer metastasis. Nakamura et al. demonstrated that EVs modulate tumor cell-peritoneal cell communications and facilitate peritoneal metastasis by transferring CD44 to peritoneal cells.[114] CD44 then promoted invasion by inducing secretion of MMP9 by peritoneal cells leading to improved cancer cell invasion.[114] Inhibiting release of EVs led to decreased ovarian cancer invasion, as CD44 could not be internalized by peritoneal cells.[114] In a similar mechanism, Yokoi et al. showed that EVs carrying MMP1 mRNA induce apoptosis in mesothelial cells leading to destruction of the peritoneal, mesothelium barrier, promoting tumor establishment.[115] mRNAs in EVs have also been associated with metastasis. Ovarian cancer cells' associated EVs have been found to carry miR-630, which, when transferred to fibroblasts, converts fibroblasts to CAFs, which thereby promotes invasion and metastasis of ovarian cancer cells.[86] The notion of EV-mediated conversion of fibroblasts to CAFs has also been reported by other investigators.[116]

EV-associated miRNA has been shown to induce chemoresistance in ovarian cancer cells. The role of various EV-associated miRNAs, including miR-21-3p, miR-21-5p, and miR-891-5p, has been explored in carboplatin resistance in ovarian cancer.[117,118] Hypoxia has also been shown to alter the composition of EVs and increase chemoresistance in ovarian cancer cells.[119] Recently, tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE-1) have been explored for their role in ovarian cancer chemoresistance, where the authors demonstrated that EVs associated with TIE-1 led to cisplatin resistance in recipient cells by decreasing the DNA damage effects of cisplatin.[120] It is also reported that, when treated with cisplatin, ovarian cancer cells released EVs, which promoted tumor cell invasion and resistance to therapy particularly when bystander cells, which are free from cellular stress, uptake the cancer cell released EVs.[121] Emerging evidence of the role of EVs in ovarian cancer chemoresistance highlights the importance of further investigation of EV-mediated immune evasion, metastasis, and chemoresistance.

Immunotherapy has been practice changing in the field of oncology, and the first tissue or site-agnostic drug approval by the US Food and Drug Administration came in 2017 for the PD-L1 inhibitor, pembrolizumab.[122] Despite significant clinical benefits for multiple disease sites, only modest anti-tumor activity was reported in ovarian cancer.[123] We have reported that SPHK1-loaded EVs increase S1P levels in the ovarian cancer TME, thereby impacting immunotherapy.[65] Our study demonstrated that T cell-mediated cytotoxicity was strengthened when SPHK1 was inhibited, and when combining SPHK1 inhibitor with anti-PD-1 antibody, anti-PD-1 therapy was more effective for ovarian cancer treatment.[65] Therefore, further characterization of EVs may provide unexpected opportunities to identify therapeutic combinations that could improve the efficacy of immunotherapy.

EVs in ovarian cancer therapeutics

EVs exhibit great therapeutic potential in gynecologic malignancies given their ability to penetrate sites of both primary tumors and the metastatic niche.[41,124] Certain properties of EVs have made them an obvious choice for drug delivery of cancer treatments. Such features are their biocompatibility, high stability in blood circulation, and high specificity to the target cells. Other than these, EVs can also tolerate the immune system and are capable of crossing the blood-brain barrier. EVs could also be loaded with small molecules, proteins, peptides, small interfering RNAs, and chemotherapeutic drugs, which can be used to attack multiple targets simultaneously (Figure 3).

There is an emerging field of engineered EV mimetics that consists of basic features of natural EVs with the additional capability of loading

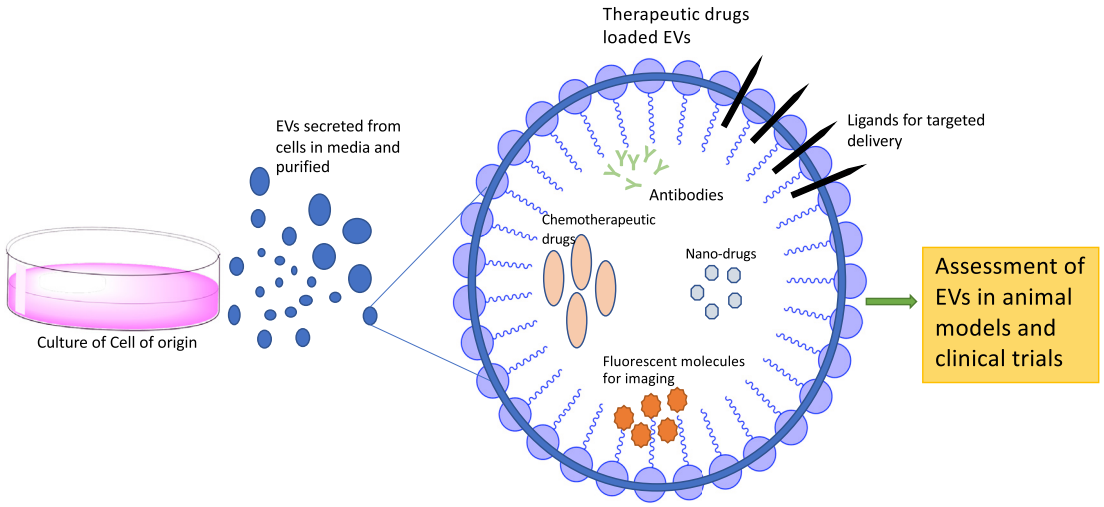

Figure 3. Schema shows the application of EVs for the delivery of targeted agents for cancer therapy

and delivering therapeutics to target cells. Pisano et al. developed a semi-synthetic immune-derived exosome mimetic, which was loaded with the chemotherapeutic agent doxorubicin.[124] When the effect of both doxorubicin-loaded immune-derived exosome mimetics and exosomes was tested on three-dimensional spheroids from the SKOV3 cell line, both encapsulated forms were more cytotoxic than free doxorubicin.[124] These exosome mimetics have great therapeutic potential as they can be effectively produced in large quantities to aid in drug delivery.[125]

Several therapeutic approaches are being developed where EVs could be employed for cancer therapy. One such approach is ExoSTING, in which an engineered EV is loaded with cyclic dinucleotide agonists of the stimulator of interferon genes (STING), which enhances the potency of cyclic dinucleotide agonists, while avoiding the systemic inflammation and failure to establish immune memory, which occurs when cyclic dinucleotide agonists are administered directly into tumors.[126] The use of ExoSTING in pre-clinical models, generated anti-tumor immunity and enhanced the therapeutic window, showcasing the benefits of novel therapies when encapsulated in EVs.[126] In a different therapeutic mechanism utilizing EVs, Baydoun et al. demonstrated, in a pilot study, that photodynamic therapy using photosensitizer pyropheophorbide α-polyethylene glycol-folic acid activates an immune response against microscopic ovarian cancer cells by way of promoting the release of EVs.[127] As these examples demonstrate, EVs are promising agents because they hold great therapeutic potential through multiple mechanism, including drug encapsulation, delivery across biological barriers, reduction of systemic inflammation, and promotion of anti-tumor immune response.

Clinical translation potential and future directions

Although the cargo of EVs appear promising, there are some major challenges before EV-associated markers can be translated usefully to women's health clinics. A major point of consideration is the step-by-step investigation of the association between biomolecules and the clinically important aspects of patient pathology. Several EV biomarkers have been shown to be differentially regulated between normal and disease conditions; however, the valid biological concentration range to show pathological conditions is not known. Researchers must also show the suitable non-invasive techniques to check the expression of theses markers in clinics so that it will be feasible to utilize this knowledge in the care of patients. Cutting-edge research and methodology including advances in nanotechnology, microfluidics, and algorithms, including machine learning, have now shown that the associated cargos of EVs have diagnostic potential utilizing small samples,[57,105,108] although the discrete functional aspects of these biomolecules in cancer are yet to be explored. Studying the functional role of EVs *in vivo* requires novel *in vivo* models, which have been created in the form of a new knockin mouse bearing a CD63-$^{emGFPloxP/stop/loxP}$ knockin cassette, which allows for labeling circulating CD63+ vesicles from any cell type when crossed with lineage-specific Cre recombinase driver mice.[128] This will enable the characterization of cell type-specific EVs *in vivo*, which should be a valuable genetic tool for generating type-specific CD63+ EVs that circulate in the serum and can be isolated and characterized using various research methodologies.[128] Besides the diagnostic potentials of EVs both *in vitro* and *in vivo*, therapeutic utilities of EVs have also become a leading area of research, poised for advances in the near future.[129]

Apart from use as biomarkers in diagnosis and prognosis, EVs are natural carriers of nano-molecules, leading to potential in drug discovery. The uniqueness of EVs in drug loading and delivery has made them a natural candidate for targeted drug delivery.[130] Therefore, learning the mechanism of EVs' synthesis, cargo sorting, in-depth characterization, and large-scale manufacturing are among the next challenges to be addressed. In 2020 the group, extracellular vesicle translation to clinical perspectives (EVOLVE) was formed to

provide "EV-specific recommendations for manufacturing, quality control, analytics, non-clinical development, and clinical trials."[131] This group documents the current landscape of clinical trials and is focused on therapeutic uses of EVs.[131] The topics of current clinical trials involving EVs range from the ability of plant EVs to deliver nutritional products to colon tumors or normal colon tissue[132,133] to treating participants with metastatic pancreatic cancer with a specific KRas mutation using mesenchymal stem cell-derived EVs loaded with small interfering RNA to target the mutation.[134] One clinical trial focusing on autologous platelet and EV-rich plasma infusion in the postoperative treatment of temporal bone cavity inflammation showed improvement in patient symptoms following treatment.[135] While the majority of clinical trials have not yet met accrual goals nor reached endpoints for analysis,[136,137] these hurdles need to be overcome to obtain sufficient clinical data to evaluate the potential of EVs for patient care.

In brief, EVs have revolutionized our current knowledge on the TME, tumor escape mechanism, and drug delivery. However, the lack of a standardized procedure for EV isolation, storage protocols, and standard assays to evaluate EV qualities are the few hurdles that need to be addressed before implementing EVs for patient care. Transmembrane proteins in EVs may be responsible for organ tropism; therefore, engineered EVs with specific proteins on the EV membrane may help EVs to reach the required organs in sufficient quantities *in vivo*. Because research on the clinical applications of EVs are in infant stages, current advances in the EV research field will facilitate the process of utilizing EVs in clinical settings in the near future.

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AUTHOR CONTRIBUTIONS

L.A.McA. and P.C.-R. conceived the topic and writing plan. L.A.McA., P.G., and R.S. performed the literature search, prepared figures, and prepared the drafts. P.C.-R. and S.P. edited the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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