Title
The diagnostic and prognostic potential of miRNAs in epithelial ovarian carcinoma

Running title
MicroRNAs in epithelial ovarian carcinoma

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Abstract/Summary
Ovarian cancer causes more than 100,000 deaths globally per year. Despite intensive research efforts there has been little improvement in the overall survival of patients over the past three decades. Most patients are not diagnosed until the cancer is at an advanced stage, by which time their chances of still being alive after five years are appallingly low. Attempts to extend life in these patients have been, for the most part, unsuccessful. This owes partly to the lack of suitable biomarkers for stratifying patients at the molecular level, into responders and non-responders. This would lead to more drugs being shown to have a clinical benefit and being approved for use in subgroups of patients. On the other hand there is also a desperate need for improved biomarkers for earlier detection of ovarian cancer; if the disease is detected sooner there is a significantly improved outlook. In this review we outline the evidence that miRNAs are deregulated in ovarian cancer, what this can tell us about tumour progression and how it could be used to improve patient stratification in clinical trials. We will also describe the potential for circulating miRNAs, both associated with proteins or carried in vesicles, to be used as diagnostics for earlier detection or as biomarkers for informing clinicians on the prognosis and best treatment of ovarian cancer.

Key Points
The transcriptional landscape of miRNAs in the blood is altered during ovarian cancer progression; levels correlate with survival and prognosis as shown by numerous studies.
miRNAs in Extracellular Vesicles in the blood also appear to reflect the characteristics of the primary ovarian carcinoma.
Hence miRNAs as well as EVs are potential biomarkers, providing a bio-accessible route for monitoring the presence and behaviour of tumours in the body.

1. Introduction

Ovarian cancer is the most deadly gynaecological cancer, with approximately 200,000 new cases diagnosed globally each year and more than 150,000 deaths due to the disease annually[1]. The overall five-year survival for ovarian cancer is approximately 45% [2]. Important reasons for this low survival rate include the acquisition of chemotherapeutic resistance frequently seen in these tumours, and the difficulty in diagnosing the disease which often leads to late presentation of the condition [3,4]. Strikingly, patients whose tumours are diagnosed in the early stages have a five-year survival rate of over 70% [5]. Epithelial ovarian carcinomas (EOC) represent by far the major type of ovarian tumour, and of these a further classification can be made on the basis of histological morphology: serous (70%, of which the majority are high grade serous carcinomas and the remainder are low grade serous carcinoma), clear cell (approx. 10%), endometrioid (approx. 7%) and mucinous carcinoma (approx. 3%) [6]. However, despite differences in their formation (both in terms of their tissue of origin and molecular causation) they are all considered essentially as one disease when it comes to treatment regime.

A number of biological molecules are being investigated for their potential in detecting ovarian cancer in earlier stages or for informing clinicians on the subtype of the disease or its likelihood to respond to different treatments. Two potential biomarkers include microRNAs (miRNAs) and extracellular vesicles (EVs). miRNAs are single-stranded RNA molecules approximately 19-23 nucleotides in length that lack protein-coding ability and are able to regulate the expression of other genes [7]. The human genome encodes more than a thousand miRNAs, and each miRNA is able to regulate the expression of multiple genes, generally via their incorporation into the RNA-induced silencing complex (RISC) and repression of target mRNA (via sequence-specific base-pairing between the miRNA and mRNA) [8]. This repression is achieved by inhibition of mRNA translation or degradation of the mRNA transcript [7]. Various experimental approaches have demonstrated that miRNAs are important regulators of key developmental processes such as cell division and differentiation, embryonic development and stress response [9-12]. It has also been shown that miRNAs deregulation can be involved in the formation of ovarian tumours and the acquisition of drug resistance in these tumours [13-18].

EVs are a type of vesicle released by cells into the extracellular space [19]. It was initially unclear what the importance and roles of EVs might be, with some suggesting that their primary function was to remove unwanted cellular material, but with a recent explosion of interest in EV biology it has emerged that they do play a variety of roles in many biological processes in cells and organisms [20-21]. Human cells can produce different kinds of EVs which can (to a certain extent) be distinguished by their size and protein composition: e.g. exosomes (approx. 40-150 nm in diameter) are released when multivesicular bodies fuse with the plasma membrane; larger microvesicles are produced by shedding of vesicles at the cell

surface; apoptotic bodies are thought to be released by cells undergoing programmed cell death. EVs can carry various types of cargo, including proteins and miRNAs, which can be delivered to other cells following uptake via a number of different endocytic pathways [22,23]. Given their importance in roles such as cell signalling, angiogenesis and modulation of the immune system, it is not surprising that EVs can be deregulated in disease, including cancer [24]. Indeed, their propensity to carry proteins and nucleic acids from their cell of origin, combined with their presence in every biological fluid tested thus far, makes them an ideal candidate as biomarkers to inform on the presence and nature of a developing tumour. In this review we will outline our current understanding of how miRNAs and EVs can be used to diagnose and characterise ovarian cancer.

2. Ovarian cancer, classification and treatment

Epithelial ovarian carcinoma (EOC), as is being discovered for so many different tumour types, is in fact a variety of diseases. These include clear cell carcinoma (CCC), mucinous carcinoma (MC), endometrioid carcinoma (EC), low grade serous ovarian carcinoma (LGSC) and high grade serous carcinoma (HGSC) [25]. Whilst there are regional variations in the proportions of each subtype, the most commonly presenting disease is HGSC. Using a broad dualistic classification the subtypes of EOCs can be divided into two main groups: type I and type II. These different subtypes differ in their origin, response to chemotherapy and prognostic outlook (table 1). Type I tumours tend to be less aggressive (the exception to this is CCC) and, although they don't tend to respond as well to chemotherapy, if diagnosed before the tumour has spread beyond the ovary the prognosis is relatively good [26-28]. Type II tumours (HGSC) are usually diagnosed in the later stages of the disease. After responding well (in most cases) to chemotherapy they tend to relapse and prognosis is rather poor [29].

Treatment for EOC usually involves surgical removal of the affected ovary (if the disease is in an early stage) or 'debulking', in which as much of the tumour is removed as possible. Debulking has been shown to improve overall survival, with the amount of residual tumour remaining following surgery one of the few factors to correlate well with survival [30,31]. Following surgery the patient is treated with platinum-based compounds, primarily carboplatin [32]. These platinum compounds form cross-links with the DNA and induce apoptosis in rapidly dividing cells. Platinum has been used as a first line treatment of EOC since the 1970s; in recent years the use of taxanes (inhibitors of microtubule dynamics) such as paclitaxel was shown to improve overall survival and is now a standard part of EOC treatment [32-34]. Recent improvements in our understanding of the molecular pathogenesis of tumours, including EOC, have led to a raft of new potential drugs which are being explored in various trials. Inhibitors of angiogenesis appear to have potential, with trials for Bevacizumab showing improvements in overall survival within some subgroups of patients [35]. The use of Poly(ADP-ribose) polymerase (PARP) inhibitors is also emerging as a potential tool to treat EOC. PARP is an enzyme involved in repairing single-strand breaks (SSBs) in DNA. In wild type cells the use of PARP inhibitors prevents the repair of single

strand breaks which are then converted to double strand breaks (DSBs) during DNA replication; these can then be repaired by the homologous recombination (HR) DNA repair machinery. However, in many EOCs the HR system is deficient, so treatment with PARP inhibitors can induce accumulation of DSBs which are toxic to the cell. This is known as synthetic lethality: the inhibition of PARP is not in itself lethal, but when combined with a mutation that affects HR it becomes lethal [36,37]. A number of PARP inhibitors are currently in trials with other first line chemotherapeutics. Whilst many of these drugs lead to improvements in progression free survival, there has been very little improvement in overall survival or cure rate over the last 30 years [38]. There is a need for better molecular understanding of EOC, so that we may uncover novel potential drugs and stratify patients and better understand those who respond better to specific treatments.

3. Molecular characterisation of EOC

The advent of high throughput genomic technologies has yielded a powerful tool for gaining better of understanding EOC. The findings from several sequencing, microarray, transcriptomic and epigenomic studies are giving new insight into the disease but also give new potential for further stratification of tumour types [39].

Sequencing studies have revealed patterns of mutation which are characteristic of type I and II EOCs. Virtually all HGSCs harbour a *TP53* mutation, and about half of them also have mutations in genes involved in the homologous recombination DNA repair mechanism [40]. Other commonly seen changes in HGSC include the presence of genomic instability and amplifications of the *CCNE1* gene [40]. Type I EOCs carry mutations in a greater variety of genes including *PIK3CA* (a subunit of the Phosphatidylinositol 3-kinase complex), *PTEN*, *ARID1A, ERBB2, KRAS* [41], *ERK and BRAF* [41], with the prevalence of these mutations different across the subtypes (table 1). The specific patterns of mutation can sometimes be linked to outcome; for example: LGSCs with a mutation in *BRAF* were found to be diagnosed at an earlier stage and had a better outcome than those with *KRAS* mutations [42]. Mutations in *BRCA1/BRCA2* are associated with better response to platinum-based treatment, longer progression-free survival and overall survival [43,44]. Increased expression of *HOXA10* in CCC is associated with reduced overall survival [45].

Type II tumours can be further classified on the basis of morphology or transcriptomic landscape. Morphologically HGSC can be divided into those with classical appearance and a second group known as the SET variant (Solid pseudoEndometrioid Transitional) in which there are more tumour-infiltrating lymphocytes and a higher mitotic index. The SET tumours are more often associated with *BRCA1* mutations, are more likely to occur in younger women but also have a better clinical outcome [46,47]. Further heterogeneity of HGSC is revealed by transcriptomic studies; analysis of gene expression in these tumours shows that the disease can be divided into four subtypes – Differentiated, Immunoreactive, Proliferative and Mesenchymal [40,48]. Subsequent studies have shown that these molecular subtypes can be

correlated with outcome, with best survival for patients with the Immunoreactive subtype but worst for the Proliferative and Mesenchymal subtypes [49]. A 193-gene transcriptional signature was also found to be a good predictor of overall survival [40] . A gene expression analysis in high grade clear cell and endometrioid carcinoma showed similar subgroupings as the high grade serous carcinoma, as well as an additional two expression subgroups associated with early stage tumours [50]. Other molecular signatures such as lin28b/let-7a/IGF-II and let-7a/*HIWI* are also shown to be associated with overall survival and prognosis [51,52]. Taken together these results underscore the potential of molecular profiling in characterising the nature of EOC.

There are numerous benefits to analysing EOCs at the molecular level. It gives new insight into potential therapeutic targets and who might benefit from them. For example, the finding that 50% of HGSCs have a defect in the HR system could be a good indicator of who might benefit from the use of PARP inhibitors [40, 53]. Indeed, the use of Olaparib, a PARP inhibitor, was recently approved for late stage patients in combination with a test for *BRCA1/2* mutations [54]. Sequencing studies have revealed activation of certain signalling pathways in some EOC subtypes, which may allow us to specifically target these pathways in the therapeutic setting. In a pre-clinical model of CCC it was shown that *EZH2* inhibitors (which target the histone methyltransferase Enhancer of Zester homolog 2) induced synthetic lethality but only when the *ARID1A* gene was compromised [55]. Finally, further molecular characterisation of EOCs gives clinicians and researchers the opportunity to stratify patients according to molecular subtypes and thus discover which patients benefit most when given a certain drug [56]. Such stratification also increases the probability that new drugs can be developed that gain approval for use in the clinic.

4. The role of miRNAs in molecular characterisation of EOC

There is a wealth of evidence emerging that miRNAs are deregulated in EOC (table 2) [57]. Whilst there are few examples of mutations in miRNAs during the development of ovarian cancer, de-regulation of expression has been observed in numerous studies [58]. Loss of miRNA function can also be achieved by epigenetic mechanisms, deletion of larger genomic regions, sequestration by pseudogenes/ ceRNA acting as endogenous sponges or blocking of miRNA maturation [40,59-62].

Early studies aimed to characterise the changes that occur in EOC compared to normal ovarian tissue. In one of the earliest studies of its kind, Iorio *et al* showed that the miR-200 family (which contains miR-141, miR-200a, miR-200b, miR-200c and miR-429) is upregulated in EOC, whilst miR-199a, miR-140, miR-145 and miR-125b-1 were down-regulated [63]. Another study showed that several miRNAs, including miR-100, miR-199a*, miR-200a and miR-214, are frequently deregulated in EOC [64].

The pattern of expression also has the potential to inform clinicians on the subtype of EOC. For example, one study showed that expression of miR-509 and miR-510 could distinguish between CCC and HGSC [65]. miR-153, miR-485-5p and miR-519a can also vary in their levels between the subtypes [66]. Others have also observed specific expression signatures for different EOC subtypes [64].

Further studies aimed to identify miRNAs whose de-regulated expression was indicative of cancer stage, prognosis, and outcome (table 3). A large scale molecular analysis of more than 400 HGSCs showed that there were three subtypes based on miRNA expression patterns [40] Survival of patients with miRNA subtype 1 was significantly longer than those with subtypes 2 or 3, again demonstrating the ability of miRNA levels to stratify patients and help to predict outcome [40].

Vecchione *et al* showed that the levels of three miRNAs, miR-484, miR-217 and miR-642 can be used to predict chemoresistance following treatment [67]. Interestingly, the link between miR-484 and chemosensitivity appears to be modulated through regulation of angiogenesis and thus the vasculature within the tumour microenvironment [67]. In another study it was shown that decreased level of miR-145 was associated with advanced stage, metastasis, lymph node involvement, earlier recurrence and worse overall survival in patients with HGSC [68,69]. Higher miR-506 levels appear to associate with better overall survival, reduced progression free survival and improved response to treatment, possibly by inhibiting expression of the double strand repair protein *RAD51* or EMT regulators [70,71].

The let-7 family of miRNAs has been implicated in a variety of cancers, including EOC. The ratio of the *HMGA2* gene over its regulator, let-7d, was associated with worse progression free survival [72]. Decreased let-7i was associated with chemoresistance and reduced progression free survival [73]. Lower levels of let-7b (and miR-199a) were associated with worse prognosis [74]. A microarray study revealed that miR-21 and miR-let7 family members were the most frequently downregulated in ovarian cancer, whilst miR-221 was up-regulated [75]. Low levels of let-7a were linked with were associated with better progression free survival, overall survival and better response to the combination of platinum and paclitaxel [76]. High methylation of the let-7a-3 gene correlates with better overall survival [77]. Indeed, miRNAs are known to affect resistance to chemotherapy via a variety of molecular mechanisms, which would ultimately impact on the overall survival of the patient [18].

In some cases the link between miRNA expression and outcome is less clear. The case of the miR-200 family provides one such example. Some studies show that its reduced expression is associated with relapse and shortened overall survival [78-81], whereas authors of other studies have concluded the opposite and that higher levels of expression are associated with worse survival [65, 74, 82]. This could be due to technical differences in sampling, processing or analysis of samples in different studies, or it could be because miR-200 family members have context-dependent roles. For example, miR-200, which is known to repress

epithelial to mesenchymal transition (EMT), could be lost during the invasive phase of tumour spread but then raised when cancer cells undergo mesenchymal to epithelial transition (MET) at a secondary site [83]. The differential effect may also come from the activity of additional factors; in one analysis of EOC tumour samples it was shown that miR-200c correlated with good or bad outcome depending on the subcellular localisation of the RNA-binding protein HuR [84]. Further study should eventually allow us to understand these conflicting results and get further insight into the role of the miR-200 family in EOC.

Results for other miRNAs also show conflicting results. In a cohort of 50 serous ovarian carcinoma samples it was seen that high levels of miR-29b were associated with reduced disease free survival [85], whilst the opposite was found in another study [86]. Similarly, miR-30d is found to be associated with worse clinical outcome in one study [87] but with better outcome in another [88]. Indeed, despite the large number of studies (not all of which have been described in the main text, though many of which are summarised in table 3) that have tried to correlate expression of miRNAs with EOC subtype, stage, grade, relapse, chemoresistance and survival, there is little consensus amongst the different studies. There are several reasons why this might be. Differences in the detection methodology, sampling and analysis methods could affect the outcome of miRNA profiling. In addition, many studies that try to compare tumour miRNA levels to surrounding healthy tissue do not accurately isolate normal epithelial but instead sample stromal tissue [89]. There are also differences in sample size and the clinical data associated with the cohorts in different studies [57]. Intra and inter tumour variability is also an issue which needs to be better understood in order to better characterise the effects of miRNAs in EOC [90]. Another variable is that the subtypes of EOC are essentially different diseases, and when combining subtypes in various analysis it can affect whether molecular markers correlate with clinical parameters [91]. In order to make experiments more comparable there needs to be a concerted effort to understand the effects of these differences on the overall quantification of miRNAs in EOC, and a greater degree of standardisation across the field.

Better understanding of miRNA deregulation will be beneficial in the long run. miRNAs represent a molecular biomarker that could directly inform treatment choice, and could help to stratify patients into groups that are more likely to respond to one treatment compared to another. For example, patients with *BRCA1/2* mutations are more sensitive to chemotherapy and also to PARP inhibitors [92]. However, only a small proportion of patients have mutations in *BRCA1/2*, yet many still respond well to treatment [40]. It is conceivable that in some cases of EOC the *BRCA1/2* genes (or any others involved in HR) are not mutated but are instead being repressed by a miRNA which is being aberrantly over-expressed. Indeed, in one study it was shown that over-expression of miR-145a, miR-148a or miR-545 (which can all target *BRCA1/2*) is associated with improved overall survival (OS) and progression free survival (PFS) in patients with wild-type *BRCA1/2* [93]. There is thus a real need to better characterise the deregulation of miRNAs in ovarian cancer.

5. Early diagnosis of EOC – the potential for miRNAs

The treatment for EOC is costly and has not improved a great deal in the last couple of decades. There is a strong argument for focusing efforts on earlier detection and diagnosis of the disease. The majority of EOC is detected once the tumour has reached a later stage. If the tumour is detected in stage I them the five-year survival rate is 93%, but this reduces to 70% in stage II and plummets to 37% and 25% in stages III and IV, respectively [94]. Better biomarkers are therefore urgently needed to reliably detect EOC before it progresses to later stages.

There is currently no useful biomarker for the early detection of cancer. Cancer antigen 125 (CA125) is known to be raised in the serum of a high proportion of patients with EOC [95], but a number of other conditions can also be associated with higher levels of serum CA125, including pregnancy, menstruation and endometriosis [96]. In some cases of EOC, particularly during early stages, the level of CA125 is not raised [97]. The use of CA125 therefore is not sufficient by itself to reach the level of sensitivity and specificity of a good diagnostic assay. Indeed, clinical trials using CA125 as a screening tool show no or modest improvements on overall survival [98,99].

A number of imaging techniques are also available for detecting or characterising EOC [100]. This includes the use of transvaginal ultrasound, but clinical trials thus far show that it is of limited value in reducing mortality due to ovarian cancer [98,99]. The combination of more than one biomarker could be a way to improve sensitivity and specificity of such tests. For example, the Risk of Malignancy Index (which combines CA125 levels, ultrasound results and menopausal status), the Risk of Ovarian Malignancy Algorithm (which combines CA125 quantification with the levels of human epididymal secretory protein 4 [HE4]) and the OVA1 test (which combines quantification of five markers: CA125, transthyretin, apoliprotein A1, transferrin and β-2 microglobulin) can all improve sensitivity and specificity compared to using CA125 levels alone [101-104]. However, no biomarker thus far identified has been able to show a positive impact on overall survival. Indeed, definitive diagnosis of disease is only really possible once the patient is examined during surgery. There is therefore a real need for improved biomarkers for diagnosis of EOC as well as for predicting outcome, informing treatment and monitoring drug response of the tumours.

The use of miRNAs as non-invasive biomarkers for EOC has been explored by several groups. miRNAs represent a good candidate in this context, as they can be excreted by cells into the extracellular environment, they can be protected against degradation by encapsulation in vesicles (see below) or via interactions with proteins, and they can be detected at relatively low levels by nucleic acid amplification methods [105].

Several groups have provided specific examples of changes to circulating miRNAs in patients with EOC (table 4). In one early study qRT-PCR was used to show that miR-21, miR-126, miR-29a, miR-92 and miR-93 were over-expressed in the serum of 28 EOC patients

compared to 15 unmatched healthy controls. Interestingly, in three of the patients with elevated miR-21/92/93 there were normal CA125 levels prior to surgery, suggesting that the use of miRNA detection could help in identifying some patients that CA125 screening does not pick up [106]. The levels of miR-145 were found to be reduced in the serum of patients with malignant EOC [107], whereas miR-221 was seen to be increased in the serum of patients compared to healthy age-matched controls [108]. Levels of miR-200a/b/c were found to be elevated in the serum of EOC patients compared to controls [109]. A miRNA signature in plasma was able to distinguish between benign and cancerous tumours and was correlated with overall survival [110]. In another study of HGSC a number of miRNAs were measured in the serum of patients, and the levels of let-7i-5p, miR-122, mIR-152-5p and miR-25-3p were found to be reduced in cancer patients compared to those with benign tumours [111]. The level of miR-212 in the serum of EOC patients was significantly higher than in controls (though curiously the levels in the tumours themselves were lower than surrounding healthy tissue [112]. Levels of miR-30c-1* were increased whereas miR-181* was under-represented in the blood patients with EOC compared to age-matched controls [113]. In a study with over 300 EOC patients the levels of plasma miR-205 were increased in patients compared to the plasma of healthy controls, whilst let-7f levels were reduced [114]. Measuring the levels of these miRNAs showed diagnostic potential, particularly for stage I tumours [114]. The presence or absence of specific miRNAs could therefore be used as a diagnostic tool.

In addition it is possible that circulating miRNAs could be used as a biomarker to inform clinicians about the nature of the tumour. A miRNA analysis of stage I EOC tumours revealed distinct miRNA profiles for the different subtypes, with levels of miR-30a/miR-30a* and miR192/194 elevated in clear cell and mucinous ovarian cancer, respectively [115]. Using qRT-PCR it was shown that miR-132, miR-26a, miR-145 and let-7b were all reduced in EOC patients [116]. Plasma levels of several miRNA could distinguish between patients with endometrioid and serous cancer [117].

Some groups have taken this further and have shown that correlations can be made between the levels of miRNAs in circulating and the clinicopathological parameters of the patient. Lower levels of serum miR-145 correlate with worse survival, for example [107]. Increased level of miR-221 is correlated with later stages of the disease and was a negative prognostic factor for the disease [108]. miR-21 was upregulated in the serum of patients with EOC and was associated with later stage, grade and worse overall survival [118]. In another study lower let-7f plasma levels correlated with worse prognosis [114]. Serum levels of miR-125b in 70 EOC patients were significantly higher compared to healthy controls and levels correlated significantly with stage, lymph node involvement and metastasis [119].

Whilst most efforts to find a suitable biomarker for EOC have focused on blood-borne markers there are other potential biofluids that could be explored. In a recent study the potential of using miRNAs extracted from urine was tested; the authors found that miR-92a is up-regulated and miR-106b down-regulated in EOC [120].

As with experiments which directly profile the levels of miRNAs in tumours, the results from studies profiling circulating miRNAs do not always show the same results. This could come down to similar reasons, including the type of EOCs being tested, stages of tumours, differences in detection method, analysis etc. One consideration with profiling circulating miRNAs is the need for a sensitive detection platform. The yields of miRNA from blood are relatively lower, and qRT-PCR methods show much better sensitivity and linearity for quantification of miRNAs compared to array-based methods [121]. There are also a number of pre-profiling factors which can affect the miRNA landscape and must be further studied to further understand the variability between studies [122]. Another issue is the method of normalisation, with some studies normalising relative to global expression [109], some using spike-in controls [114] and others using specific reference genes [110,111] (the latter of which also require further characterisation and reply on the potentially false assumption that they are expressed equally across all patients).Further optimisation and standardisation is needed to make the results of different studies more comparable and to make the use of a miRNA-based diagnostic more realistic. Whilst there is no FDA-approved diagnostic to date, there is a great deal of potential for miRNAs in this realm, particularly when used in combination with established markers such as CA125 [114].

6. The potential of extracellular vesicles in EOC diagnostics

Extracellular vesicles (EVs) are known to carry and deliver different kinds of nucleic acid, including miRNAs [23, 123]. Profiling the number and content of EVs is emerging as a realistic diagnostic tool for EOC. Levels of EVs in the blood have been seen to be increased in patients with EOC [124,125] and the levels correlate positively with tumour stage [125].
The first study looking at miRNA in EVs from ovarian cancer patients showed that the elevated levels of miR-21, miR-141, miR-200a/b/c, miR-203, miR-205 and miR-214 observed in EOC was also reflected in the serum exosomes of the same patients [125]. In a more recent study serum EVs were extracted and the levels of miR-200a/b/c and miR-373 were measured by qRT-PCR [124]. Levels of all three were significantly higher in EOC patients and miR-200a/miR-373 was correlated with stage and lymph node involvement whilst miR-200b/c were associated with lower overall survival [124]. In another study the vesicular miRNA content of exosomes isolated from pleural or peritoneal effusions was measured [126]. The levels of 11 miRNAs could be associated with effusion site and tumour stage; in addition the levels of miR-21, miR-23b and miR-29a were associated with worse progression-free survival and miR-21 levels correlated with worse overall survival [126].

An issue with using EVs from plasma or serum is that the population of vesicles is a result of many cell types across the body and not just the tumour. Some good correlation between miRNA levels and tumours can sometimes be seen, which may reflect the large increase in vesicle content seen in the circulation of patients [124,125]. Interestingly, in a pilot study using urinary exosomes there were no detectable differences in the levels of miRNAs in either endometrial cancer or HGSC compared to controls [120]. Whilst it is still quite early

for the field of EV markers in the diagnosis/prognosis of EOC there is a great deal of potential in this area.

7. Conclusions and outlook

The last 30 years has seen a large advancement in our understanding of the molecular basis of EOC, though this has yet to be translated into improved overall survival for patients. Although new drugs are being developed their testing via clinical trials is not optimal, in part due to the lack of enough biomarkers for stratifying patients. miRNAs have emerged as important regulators of gene function and potential biomarkers for informing treatment and prognosis. The use of circulating miRNAs is particularly exciting, as they provide a more bio-accessible route for monitoring presence and behaviour of any tumours in the body. miRNAs appear to be well protected in the circulation and numerous studies show that the transcriptional landscape in the blood is altered during tumour progression. However, their potential clinical use is tempered by the current practical realities – there is little consensus between studies and much work needs to be done to establish specificity and sensitivity of such tests in the hands of different labs. The lack of consensus most likely comes from differences in sampling techniques, cohort sizes, detection platform, analysis methods etc. This has prevented the development of a meaningful diagnostic based on circulating miRNAs. That being said it should be remembered that screens using approved biomarker tests, such as those for CA125, have not yielded any improvement in diagnosis or overall survival to patients. Perhaps the best hope for a miRNA-based diagnostic test in the short to medium term would be the combination of miRNA detection with CA125 to improve sensitivity and specificity [114]. In some cases patients presenting with low CA125 have deregulated miRNAs, suggesting that combined detection of CA125 could help clinicians to pick up early cases of EOC before they are detected by conventional means [106]. In the long term the methodology must be improved and standardised, but there is massive potential for the use of miRNA profiling as both biomarkers of tumour behaviour and treatment response as well as early detection.

1 Table 1: Characteristics of subtypes of Ovarian Cancer

	Type II	Type I		
	High grade serous	**Low grade serous**	**Endometrioid**	**Clear cell**
Origin	Fallopian Tube	Fallopian Tube	Endometriosis	Endometriosis
Diagnosis	Usually detected at a later stage after metastatic spread	Usually detected early	Usually detected early	Usually detected early
Proportion of all Ovarian Cancers [6]	Approximately 70% most of which are HGSC		Approximately 7%	Approximately 10%
Grade	High grade	Low grade	Low grade	High Grade
proliferative capacity	High proliferative activity	Low proliferative activity	Low proliferative activity	Low proliferative activity
Response to chemotherapy	Good response but often recurs	Metastatic tumours often chemo-resistant	Metastatic tumours often chemo-resistant	Poor response to chemotherapy
prognosis	Poor prognosis if diagnosed in later stages	Better prognosis than HGSC	Better prognosis than HGSC	Some studies show a worse prognosis than HGSC
Commonly mutated genes	HR deficiency, p53 mutations, *PI3K/RAS*, NOTCH signalling	*PIK3CA, BRAF, KRAS*	*ARID1A*, *PIK3CA*, *PTEN, MMR* deficiency	*ARID1A, PIK3CA*
genetic instability	High	Low	Low	Low

2 Abbreviations: HGSC – High grade serous Carcinoma, HR – Homologous recombination

3

4 Table 2: microRNAs differentially expressed in ovarian cancers to benign or normal ovarian tissue

First Author	Sample Size	Detection Method	Ovarian Carcinoma Vs Normal
Lee[88]	171 – 109 OC, 22 normal, 17 benign and 23 borderline	qRT-PCR assays	↑miR-30c, miR-30d, miR-30e-3p, ↓miR-370 in carcinomas compared to benign ovarian tissues
Vilming Elgaaen[65]	35 HGSC, 19 CCC	affymetrix, qRT-PCR	↑miR-205-5p, miR-200 family members and miR-182-5p; ↓miR-383 in HGSC compared to OSE
Nam[74]	28 samples - 20 SOC, 8 normal	microarray, northern blot	↑miR-200c, miR-93, and miR-141; ↓let-7b, miR-99a, and miR-125b were down-regulated in serous carcinoma
Iorio[63]	84 - 69 OC and 15 normal	microarray, northern blot and qRT-PCR	39 miRNA signature including ↑miR-200a and miR-141 and ↓miR-199a, miR-140, miR-145, and miR-125b1 in cancer
Cao[82]	100 - 50 OC and 50 normal	qRT-PCR	↑miR-200a, miR-200b and miR-200c in ovarian cancer compared to normal tissue
Dahiya[75]	34 OC	microarray	25 upregulated and 31 downregulated miRNAs between control and cancer tissues; miR-21 and let-7 most frequently downregulated; miR-221 upregulated
Laios[128]	28 OC	TaqMan® MicroRNA Assay Human Panel Early Access kit, qRT-PCR	↑miR-223 and ↓miR-9 associated with recurrence of ovarian cancer
Vecchione[67]	198 SOC	microarray, Taqman microRNA assay	↓miR-484, miR-217 and miR-642 downregulated in chemoresistant tumours
Chao[81]	176 OC and 20 benign	TaqMan MicroRNA Assays Human Panel Early Access kit	↑miR-187 and miR-200a in ovarian cancer tissues than benign tissues
Kim[68]	74 HGSC and 10 normal fallopian tubes	qRT-PCR	↓miR-145 in HGSC as compared to normal

Dong[69]	48 HGSC and 19 normal fimbriae	qRT-pPCR	↓miR-145 in HGSC as compared to normal
Jin [129]	100 EOC and 10 normal tissues	qRT-PCR	↓miR-150 in epithelial ovarian cancer compared to normal tissue
Chen[130]	94 OC, 44 benign or normal	qRT-PCR	↓miR-106b in ovarian carcinomas as compared to normal tissues or benign ovarian disease
Yang[64]	30 OC and 10 normal tissues	northern blot	↑miR-200a, miR-199a and miR-214 and ↓miR-100
Corney[131]	83 EOC	qRT-PCR	↓mir-34 a/b*/c decreased in EOC with mutant p53; ↓miR-34a also in EOC with wild type p53
Dai[86]	160 OC and 30 normal	qRT-PCR	↓miR-29b in ovarian cancer
Lee[88]	171 - (109 OC)	Taqman PCR assays	↑miR-30c, miR-30d, miR-30e-3p and ↓expression of miR-370 in ovarian carcinomas and benign tumours
Wan[132]	109 OC with adjacent normal tissue	qRT-PCR	↓miR-22 in cancer tissue as compared to normal tissues
Kim[66]	103 – 54 OC	microarray	↑miR-519a and ↓miR-153 and miR-485- 5p in ovarian carcinomas as compared to benign and borderline tumours
Wei[112]	60 OC with adjacent normal tissue	qRT-PCR	↓miR-212 in ovarian cancer compared to normal tissue

Abbreviations: CCC – Clear Cell carcinoma, EOC – Epithelial Ovarian Carcinomas, HGSC – High Grade Serous Carcinoma, OC – Ovarian Carcinoma, qRT-PCR – quantitative Real Time Polymerase Chain Reaction, SOC – Serous Ovarian Carcinoma

9 Table 3: microRNAs associated with survival and prognosis

First Author	Sample Size	Detection Method	Survival Correlation
Lee[88]	171 – 109 OC, 22 normal, 17 benign and 23 borderline	qRT-PCR	↑miR-181d, miR-30c, miR-30d, miR-30e-3p correlated with better disease free or OS
Vilming Elgaaen[65]	35 HGSC, 19 CCC	affymetrix, qRT-PCR	↑miR-200c associated with short PFS and OS
Bell (TCGA) [40]	489 OC		subtype 1 longer survival
Maarchini [78]	144 OC	microarray; qPCR	↑miR-200c correlated with OS
Hu[79]	55 with advanced OC	qRT-PCR	↑miR-200a correlated with better OS
Nam[74]	28 samples - 20 SOC vs 8 normal ovarian tissue	microarray, northern blot	↑miR-200a, miR-200b, miR-200c, miR-141, miR-18a, miR-93, and miR-429, and ↓ambi-miR-7039, let-7b, and miR-199a were significantly correlated with decreased PFS and OS
Prislei[84]	220 OC	nanofluidic genetic analyzer and a 48.48 chip array	↑miR-200c correlates with good or bad outcome depending on the subcellular localisation of RNA binding protein HuR
Cao[82]	100 - 50 normal, 50 OC	qRT-PCR	↑miR-200a, miR-200b and miR-200c correlated with shorter OS
Leskela[80]	72 OC	qRT-PCR	↓miR-429 is associated with worse PFS and OS
Shell[72]	53 OC	qRT-PCR	ratio of HMGA2 over let-7d associated with worse PFS
Yang[73]	69 OC	microarray, qRT-PCR	↓let-7i expression associated with chemoresistance and reduced PFS
Lu[76]	211 OC	Taqman miRNA assay	↓let-7a expression levels indicate a better response to combination of platinum and paclitaxel with better PFS and OS
Laios[128]	28 OC	TaqMan® MicroRNA Assay Human Panel Early Access kit, qRT-PCR	↑miR-223 and decreased miR-9 associated with recurrence of ovarian cancer
Vecchione[67]	198 SOC	TaqMan Array Human MicroRNA Set v2.0, Taqman microRNA assay	↓miR-484, miR-217 and miR-642 in chemoresistant tumours

Chao[81]	176 OC and 20 benign	TaqMan MicroRNA Assays Human Panel Early Access kit, Taqman microaRNA assays	paradoxically ↓miR-187 and miR-200a associated with poorer OS and poor prognosis
Bagnoli[133]	56 - training set, 53- test set, 50- validation set	microarray, TaqMan microRNA assays	lower expression of a cluster of 8 mirnas at chrXq27.3 associated with quicker relapse
Kim[68]	74 HGSC and 10 normal fallopian tubes	qRT-PCR	↓miR-145 correlates with advanced stage, metastasis, lymph node involvement, earlier recurrence, worse OS
Liu[70]	468 OC and 130 OC	Bioinformatic analysis of the microarray data from TCGA data and Bagnoli dataset	↑miR-506 correlated with better OS and longer PFS
Sun[71]	204 OC	in situ hybridisation	↑miR-506 better OS, longer PFS and improved response to treatment
Jin[129]	100 EOC and 10 normal tissues	qRT-PCR	↓miR-150 correlated with later stage, poor prognosis with shorter PFS and OS
Chen[130]	94 OC, 44 Benign/ normal	qRT-PCR	↓miR-106b negatively associated with stage of the tumour
Corney[131]	83 EOC	stem loop qRT-PCR	↓miR-34b* and miR-34c expression associated with later stage
Dai[86]	160 OC and 30 normal tissues	qRT-PCR	↓miR-29b correlated with later stage; along with MAPK10- and ATG9A-positivity, ↓miR-29b correlated with decreased PFS and OS
Li[87]	330 OC	ISH	↑miR-30d expression associated with lower OS
Lee[88]	171 - (109 OC)	Taqman PCR assays	↑miR-181d, miR-30c, miR-30d, and miR-30e-3p was associated with significantly better disease-free or OS
Eitan[134]	57 OC	miRNA microarray	↑miR-23a, miR-27a associated with poorer OS and disease free survival, ↑miR-449b and miR-24-2* associated with poorer OS while ↑ miR-21 associated with poor PFS
Wan[132]	109 OC with adjacent normal tissue	RT-PCR	↓miR-22 associated with lower PFS and OS

Kim[68]	103 – 54 OC	mirna microarray	↑ miR-485-5p and ↓ miR-153 associated with advanced stage, ↑miR-519a associated with poor PFS
Wei[112]	60 OC with adjacent normal tissue	qRT-PCR	↓miR-212 associated with later stage, metastasis and lymph node involvement
Wurz[135]	49 OC	Taqman microRNA assay	lower ratio of expression of miR-222 to miR-221 associated with worse OS
Gu[93]	317 HGSC	data analysis from data in TCGA	↑ miR-146a, miR-148a and miR-545 associated with better OS and PFS in patients with wild-type BRCA1/2

Abbreviations: CCC – Clear Cell carcinoma, EOC – Epithelial Ovarian Carcinomas, HGSC – High Grade Serous Carcinoma, ISH – in-situ hybridisation, OC – Ovarian Carcinoma, OS – overall survival, PFS – progression free survival, qRT-PCR – quantitative Real Time Polymerase Chain Reaction, SOC – Serous Ovarian Carcinoma

15 Table 4: MicroRNAs as biomarkers in serum/ effusions

First Author	Sample	Sample Size	Detection Method	Mirna Deregulation
Resnick[106]	serum	28 EOC vs 15 unmatched healthy controls	qRT-PCR	↑ miR-21, miR-92, miR-93, miR-126 and miR-29a and ↓miR-155, miR-127 and miR-99b in serum from ovarian cancer patients
Liang[107]	serum	270 - 84 OC, 51 benign tissues, 135 healthy controls	qRT-PCR	↓miR-145 in patients with ovarian cancer as compared with healthy controls or benign disease; also correlated with shorter OS
Hong[108]	serum	96 OC, 35 healthy controls - age matched	qRT-PCR	↑miR-221 in patients with EOC; correlated with OS
Xu[118]	serum	94 OC and 40 healthy volunteers	qRT-PCR	↑miR-21 in EOC than in healthy controls; associated with worse OS
Guo[136]	serum	50 EOC, 50 healthy controls	qRT-PCR	↑miR-92 in patients with EOC
Kan[109]	serum	28 EOC and 28 healthy controls	qRT-PCR	↑miR-200a, miR-200b and miR-200c in EOC patients serum
Shapira[110]	plasma	42 SEOC, 36 benign tumours and 23 healthy controls	qRT-PCR with ABI Taqman OpenArray system	↑ miR-1274a, miR-625-3p, and miR-720 while 19 miRNAs showed decreased plasma levels in ovarian cancer patients; ↓miR-720, miR-20a and ↑miR-223, miR-126-3p and miR-1290 were associated with shorter OS
Langhe[111]	serum	25 OC, 25 benign tumour	qRT-PCR	↓let 7i-5p, mir-122, miR-152-5p, miR-25-3p in ovarian cancer
Wei[112]	serum	60 OC and 60 healthy volunteers	qRT-PCR	↑miR-212 in EOC as compared to healthy controls
Hausler[113]	blood	24 OC and 15	microarray	↑miR-30c1* and ↓miR-342-3p, miR-181a* and miR-450b-5p in ovarian

		controls		cancer
Chung[116]	serum	18 OC and 12 controls	microarray, qRT-PCR	↓miR-132, miR-26a, let-7b, and miR-145 in ovarian cancer as compared to controls
Zuberi[119]	serum	70 OC and 70 healthy volunteers	qRT-PCR	↑miR-125b in ovarian cancer; however ↑miR-125b were associated with early stage, no lymph node involvement and no metastasis
Suryawanshi[117]	plasma	14 EAOC, 21 SOC	qRT-PCR	Expression signature of ↑miR-16, miR-191, and miR-4284 SOC compared to healthy controls, combination of miR-16, miR-21, and miR-191 can differentiate between healthy controls and EAOCs
Zheng[114]	plasma	360 OC and 200 healthy controls	taqman PCR array; qRT-PCR	↑miR-205 and ↓let-7f in plasma in EOC, ↑miR-483-5p associated with later stage ovarian cancer; ↓let-7f correlated with shorter PFS
Ji[137]	serum	62 - 31 OC, 23 benign, and 8 normal	qRT-PCR	↑ miR-93, miR-22
Zavesky[120]	urine	14 OC, 25 controls	qRT-PCR	↑ miR-92a, ↓ miR-106b downregulated
Vaksman[126]	EVs in pleural/ peritoneal effusion	86 OC	miRNA Taqman assays	↑ miR-21, miR-23b and miR-29a associated with poor PFS, ↑miR-21 associated with poor OS
Taylor[125]	EVs in blood	50 OC, 10 control	microarrays	↑miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR- 214 in EOC
Meng[124]	EVs in serum	163 EOC, 20 benign and 32 healthy controls	taqman microRNA assay and ELISA	↑miR-373, miR-200a, miR-200b and miR-200c in EOC, ↑miR-200b and miR-200c levels also associated with shorter OS

Abbreviations: EAOC – Endometriosis associated Ovarian Cancer, OC – Ovarian Carcinoma, OS – overall survival, PFS – progression free survival, qRT-PCR – quantitative Real Time Polymerase Chain Reaction, SOC – Serous Ovarian Carcinoma

Compliance with Ethical Standards

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