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REVIEW

MicroRNAs in extracellular vesicles: potential cancer biomarkers

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Extracellular vesicles (EV) are small membrane-bound structures that are secreted by various cell types, including tumor cells. Recent studies have shown that EVs are important for cell-to-cell communication, locally and distantly; horizontally transferring DNA, mRNA, microRNA (miRNA), proteins and lipids. In the context of cancer biology, tumor-derived EVs are capable of modifying the microenvironment, promoting tumor progression, immune evasion, angiogenesis and metastasis. miRNAs contained within EVs are functionally associated with cancer progression, metastasis and aggressive tumor phenotypes. These factors, along with their stability in bodily fluids, have led to extensive investigations on the potential role of circulating EV-derived miRNAs as tumor biomarkers. In this review, we summarize the current understanding of circulating EV miRNAs in human cancer, and discuss their clinical utility and challenges in functioning as biomarkers.
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INTRODUCTION

Extracellular vesicles (EV) are small membrane-bound vesicles that are secreted by various cells. EVs have been isolated from numerous cell types, including immune,[1–3] stem,[4,5] nervous system,[6,7] epithelial,[8] fibroblast,[9] keratinocyte[10] and a variety of tumor[11] cells. EVs were originally described as vesicles that were released into the extracellular space from multi-vesicular bodies (MVBs) of reticulocytes.[12,13] They were initially considered to be a mechanism by which cells discard unnecessary molecules into the extracellular space.[14–16] However, more recent studies have shown that EVs are an important mechanism by which cells communicate both locally and distantly, by transferring proteins, lipids and nucleic acids including DNA, mRNA and microRNA (miRNA).[16] In the context of tumor biology, accumulating evidence suggests that EVs play an important role in communication between tumors and the microenvironment; by transferring EV cargo, tumor cells are able to alter the function of both local and distant normal cells, thereby promoting tumor growth and metastasis.[17]

miRNAs are small RNA molecules that regulate the expression of protein-coding genes by directly binding to target mRNAs in a sequence-specific manner.[18] Bioinformatics algorithms predict that miRNAs regulate $>60\%$ of the protein-coding genes in the human genome.[19] After the discovery of miRNA transfer between cells via EVs,[20–22] EV miRNAs garnered increasing attention from the biomedical research community. Due to the stability of EV miRNAs in body fluids, and their functional association with tumor progression, circulating EV miRNAs are now extensively investigated for their potential use as cancer biomarkers. In this review, we briefly describe the functional significance of EV miRNAs in cancer biology, summarize circulating EV miRNA biomarker studies for human malignancies, and discuss their utility and limitations as circulating biomarkers.

EV BIOGENESIS

EVs are classified as either exosomes or microvesicles, as a function of their biogenesis. Exosomes are released on MVB fusion with the plasma membrane; whereas microvesicles are released by direct budding from the plasma membrane (Figure 1).[16,23,24] Exosomes are slightly smaller in diameter (~30–150 nm) and have ‘cup-shaped’ morphology when observed under transmission electron microscopy; on the other hand, microvesicles are more heterogeneous in both size (~100–1000 nm) and shape.[24]

Exosome biogenesis begins during the early-endosome maturation process (Figure 1). Early-endosomal membranes invaginate to create intraluminal vesicles (ILVs), thereby forming MVBs. There are several described mechanisms that mediate ILV/MVB formation (reviewed in[23]). The endosomal-sorting complex required for transport (ESCRT) is the best characterized mechanism, although other ESCRT-independent mechanisms also exist, which require the tetraspanin CD63 or lipid metabolism enzymes, such as neutral sphingomyelinase (nSMase)[25] and phospholipase D2 (PLD2).[26] The majority of MVBs are degraded by fusion with lysosomes; however, some populations of MVBs migrate towards and fuse with the plasma membrane, releasing their ILVs—now called exosomes—into the extracellular space. Although the mechanisms of exosome

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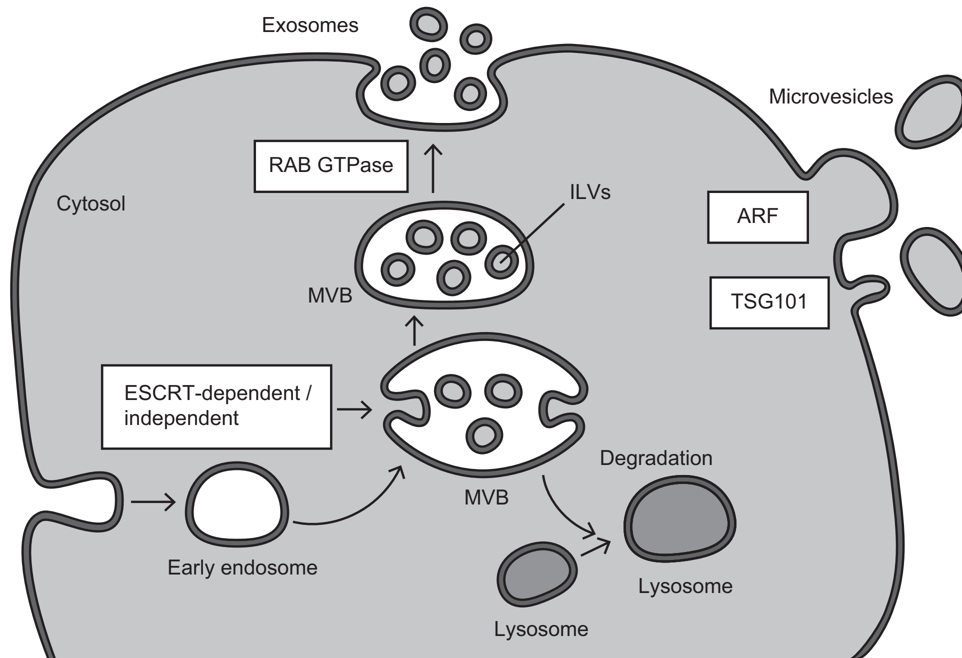

Figure 1 A schematic illustration of exosome and microvesicle biogenesis. Exosome biogenesis begins with the formation of MVBs via early-endosomal membrane invagination to create ILVs. Both ESCRT-dependent and -independent mechanisms are implicated in ILV/MVB formation. Although the majority of MVBs are degraded by fusion with lysosomes, some populations of MVBs fuse with the plasma membrane, releasing exosomes into the extracellular space. Exosome secretion involves RAB GTPases (RAB11, RAB27A/B and RAB35). Microvesicles are released by direct budding from the plasma membrane, and involve ARF1, ARF6 and TSG101. ARF, ADP-ribosylation factor, ESCRT, endosomal-sorting complex required for transport, ILV, intraluminal vesicle, MVB, multi-vesicular body, TSG101, tumor susceptibility gene 101.

secretion have not yet been fully elucidated, RAB GTPases are known to be intricately involved. Savina *et al.*[27] demonstrated that disruption of RAB11 via overexpression of its dominant-negative mutant resulted in inhibition of exosome release from the K562 erythroleukemia cell line. Ostrowski *et al.*[28] demonstrated that knockdown of RAB27A or RAB27B resulted in effective reduction in exosome secretion by performing a shRNA screen for 59 members of the Rab GTPase family in HeLa cells. In another report, inhibition of RAB35 function led to intracellular accumulation of endosomes and impaired exosome secretion.[29] Importantly, although RAB11, RAB27A/B and RAB35 play important roles in exosome secretion, the inhibition of just one of these RAB GTPases is insufficient for complete inhibition of exosome secretion.[27–29] This therefore suggests the existence of distinct populations of ILVs and MVBs within the cell, requiring unique machinery for exosome secretion. Indeed, RAB27A inhibition in the mouse mammary carcinoma cell line 4T1 resulted in a decrease in exosome secretion for certain populations (CD63, TSG101, Alix and HSC70), while not affecting the secretion of others (CD9 and MFGE8).[30]

The biogenesis of microvesicles is less well-characterized (Figure 1). Muralidharan-Chari *et al.*[31] found that the ADP-ribosylation factor 6 (ARF6) GTP/GDP cycle regulated the release of protease-loaded microvesicles from the LOX melanoma cell line. Similarly, ARF1 was found to be involved in the secretion of microvesicles in the MDA-MB-231 breast cancer cell line.[32] Nabhan *et al.*[33] demonstrated that TSG101, a component of ESCRT, regulated microvesicle secretion. These authors also showed that interaction of TSG101 with arrestin domain-containing protein 1 (ARRDC1) resulted in relocation of endosomal TSG101 to the plasma membrane, mediating release of microvesicles. Hence, insofar as some mechanisms are emerging in the differential biogenesis of exosomes vs microvesicles this is merely the beginning of an exciting field; future studies will be necessary to reveal further insight into the mechanism of microvesicular biogenesis.

EV ISOLATION METHODS

A number of EV isolation methods have been developed for the purification of exosomes and microvesicles from body fluids and/or cell culture media. The most commonly used procedure employs a series of differential centrifugation steps.[34] Following removal of cells and cell debris using low-speed centrifugations (300*g* and 2000*g*), the supernatant is spun at 10 000*g* to pellet microvesicles. Exosomes are subsequently pelleted from the supernatant via ultracentrifugation (100 000*g*).[34] Although this protocol is straightforward and frequently used, contaminants (for example, protein aggregates) can also be co-precipitated. To overcome this problem, an additional density gradient centrifugation purification step is often combined with the differential centrifugation. Exosomes are then isolated from a buoyant density of 1.08–1.22 $\mathrm{g\,ml^{-1}}$ on sucrose or iodoxanol (OptiPrep, St Louis, MO, USA) gradients.[1]

An alternate EV purification method is immunoaffinity isolation using antibody-coated magnetic beads, which allows for the selection of a more restricted population of exosome. This method has been used for the isolation of HER2-positive exosomes from the culture media of breast cancer cell lines, exosomes from the ascites of a patient with advanced ovarian cancer[35] and EpCAM-positive exosomes from the serum of patients with lung or ovarian cancer.[36,37] A common challenge with immunoaffinity isolation is the identification of exosome-specific markers. Recently, polymer-based exosome precipitation solutions have been developed (for example, ExoQuick, System Biosciences, Palo Alto, CA, USA) and widely used as a simple and rapid exosome isolation method, resulting in high yields of

proteins and RNAs.[38] It is unclear however, whether these polymer-based methods are capable of isolating pure exosomes.

To date, there are no reliable methods for purifying and discriminating between exosomes or microvesicles. Therefore, careful consideration of EV isolation methods must be taken into account when interpreting study results.

COMPOSITION OF EVS

EVs contain many biomolecules, including proteins, lipids, DNA, mRNAs and non-coding RNAs, such as miRNAs.[39] Among these, proteins are the best characterized EV cargo. The membrane protein composition of microvesicles resembles the parental cell more closely than that of exosomes.[16] Exosomes, on the other hand, are enriched with components of endosomes and proteins found in the parental cell that are involved in MVB formation, such as tetraspanins (CD9, CD63 and CD81), Alix, flotillin, TSG101 and RAB GTPases.[23] Mass spectrometry studies have shown that exosomes are enriched with proteins from the cytosol and plasma membrane, but lack proteins from the nucleus, endoplasmic reticulum, Golgi and mitochondria.[40,41] Some of the protein contents from EVs overlap between exosomes and microvesicles, thus limiting the distinction between EV types based on an individual protein.[23,42] Recent studies have used a combination of several protein markers to distinguish exosomes from microvesicles. The International Society for Extracellular Vesicles (ISEV) suggested that to claim the successful isolation of exosomes, a combination of at least three protein markers must be used,[43] including: (a) the presence of transmembrane or lipid-bound extracellular proteins (CD9, CD63, CD81, cell adhesion molecules, growth factor receptors, heterotrimeric G proteins, integrins or MFGE8); (b) the presence of cytosolic proteins (TSG101, annexins, RAB GTPases or syntenin); and (c) the absence of intracellular proteins (from endoplasmic reticulum, Golgi, mitochondria, nucleus or Argonaute/RNA-induced silencing complex (RISC)).[43]

RNA is another biomolecule that is carried within EVs. Valadi *et al.*[44] first identified the presence of mRNAs and miRNAs within the exosomes from human and mouse mast cells; these exosomes were markedly stable following treatment with RNase or trypsin. Notably, the authors also found that the mRNA contained within the mouse exosomes could be internalized into human mast cells, resulting in the production of protein from these mouse mRNAs in the recipient cells, suggesting that exosome mRNA remains functional and can be translated in other cells. This was the first identification of gene-based communication between mammalian cells. Subsequently, bioanalyzer analyses showed that exosomes and microvesicles have distinct RNA profiles.[45,46] Exosomal RNAs are generally enriched for small RNAs, including miRNAs, and lack ribosomal RNA. Interestingly, the RNA expression profiles are distinct between exosomes and their producer cells. Ohshima *et al.*[47] found high exosomal let-7 family expression in the metastatic gastric cancer cell line AZ-P7a, but lower exosomal expression in several cell lines with higher intracellular let-7a expression. Lunavat *et al.*[45] compared the RNA profiles between cells and different EV populations in the melanoma cell line MML-1 using next generation RNA sequencing. The authors found that out of 252 miRNAs that were detected, 113 miRNAs were shared between the EV sub-types and parental cells, whereas 23 miRNAs were detected exclusively in the exosomes. These observations suggest that cells have sorting mechanisms allowing for preferential secretion of specific miRNAs into the exosomes. Several mechanisms of miRNA sorting into exosomes have been reported (reviewed in[48]) such as nSMase2-dependent pathway,[20] sumoylated heterogeneous nuclear ribonucleoprotein (hnRNP)-dependent,[49] 3′-end of the miRNA sequence-dependent,[50] as well as the RISC-related pathways.[51]

EV MIRNAS IN CANCER

EVs play important roles in physiological processes, such as immune system regulation, blood coagulation, stem cell and nervous system maintenance, as well as in pathologic states such as cancer.[16,17,23] In the context of cancer biology, numerous studies have shown that tumor-derived EVs are capable of modifying the microenvironment to facilitate tumor progression, angiogenesis and metastasis.

Angiogenesis is essential for tumor development and metastasis, and tumor-derived EV miRNAs affect endothelial cells to promote this process.[52–54] Exosomal miR-92a, derived from the leukemia cell line K562, was found to be transferred into endothelial cells, resulting in enhanced endothelial cell migration and tube formation.[52] In addition, exosomal miR-135b, derived from hypoxia-resistant melanoma cells, enhanced endothelial tube formation under hypoxia by targeting factor-inhibiting hypoxia-inducible factor 1 (FIH-1).[53] Zhuang *et al.*[54] showed that SK23 melanoma cell-derived miRNAs, including miR-9, were transferred to endothelial cells largely via EVs, which they demonstrated using a transwell tumor–endothelial cell co-culture system. Functional studies showed that SK23-derived EVs led to enhanced migration in endothelial cells; this effect was attenuated by anti-miR-9 transfection, suggesting that EV-mediated miR-9 transfer was important for angiogenesis in this context.

Tumor-derived EV miRNAs have been shown to enhance metastasis by modifying the tumor microenvironment and promoting mesenchymal-to-epithelial transition (MET). Fong *et al.*[55] identified that breast cancer cell-secreted EV miR-122 facilitated tumor metastasis by modifying glucose metabolism in the pre-metastatic niche environment. Zhou *et al.*[56] showed that breast cancer-secreted EV miR-105 induced vascular permeability in endothelial cells by targeting the cellular tight junctions. Mice developed more metastases when pretreated with EVs secreted by the highly metastatic MDA-MB-231 cells, which contained high levels of miR-105. In both studies, therapeutic administration of anti-miR-122 or anti-miR-105, respectively, with tumor-derived EV injection resulted in suppression of brain and lung metastasis. MET is important for the population of metastatic sites, and the miR-200 family is a well-known mediator of this process.[57] Le *et al.*[58] showed that EV miR-200 conferred the ability to colonize lung metastatic sites via promotion of MET, using a highly metastatic breast cancer model.

EV MIRNAS AS CANCER BIOMARKERS

Given the role of tumor-derived EV miRNAs in tumor progression and metastasis, it is logical to investigate the role of EV miRNAs as biomarkers. EVs are known to exist in many types of bodily fluids, including blood,[59] urine,[60] saliva,[61] breast milk,[62] amniotic,[63] ascites[64] and cerebrospinal fluids.[65] EV miRNAs have been primarily examined for their presence in the plasma and serum of cancer patients for diagnostic or prognostic purposes. A summary of circulating EV miRNAs identified in various cancer types, detected using either miRNA profiling studies or individual miRNA expression studies are shown in Tables 1 and 2, respectively. Important caveats for these Tables and in making comparisons include: (a) the varying terms used to define EVs; (b) sample type from which the EVs were derived (that is, plasma or serum); and (c) methodological differences in EV isolation, miRNA profiling and expression normalization.

The ISEV suggests the use of plasma rather than serum as a source for EV RNA for the purpose of biomarker studies,[66] as platelets release

Table 1 Profiling of EV-derived miRNAs in cancer

Cancer	*Sample source*	*Isolation method*	*Profiling method*	*Normalization method*	*Cohort*	*Deferentially expressed miRNAs*[a]	*Ref*
Chronic lymphocytic leukemia	Plasma	Differential centrifugation	nCounter miRNA Expression Assay (NanoString, Seattle, WA, USA)	Geometric mean of the most stable 20 miRNAs	69 patients with chronic lymphocytic leukemia and 15 healthy controls	Upregulation: miR-150, miR-155, and miR-29 family members Downregulation: miR-223	93
Colorectal cancer	Serum	Differential centrifugation	3D-Gene Human miRNA Oligo Chip (Toray, Tokyo, Japan)	Amount of total RNA input	4 colorectal cancer patients with recurrence and 2 without recurrence	miR-19a, miR-19b, miR-23a, miR--92a, miR-320a and miR-4437	94
Colorectal cancer	Serum	Differential centrifugation	Human miRNA oligonucleotide microarray version 3.0 (Agilent Technologies, Mississauga, ON, Canada)	Total signal intensity of the array	88 patients with colorectal cancer and 11 healthy controls	let-7a, miR-1224-5p, miR-1229, miR-1246, miR-1268, miR-1290, miR-1308, miR-150, miR-181b, miR-181d, miR-1915, miR-21, miR--223, miR-23a, miR-483-5p and miR-638	83
Esophageal cancer	Serum	ExoQuick	TaqMan OpenArray Human microRNA panel (Applied Biosystems, Foster City, CA, USA)	Ratios of two miRNAs	18 patients with esophageal adenocarcinoma, 10 with Barrett's esophagus and 19 healthy controls	RNU6-1/miR-16-5p, miR-25-3p/miR-320a, let-7e-5p/miR-15b-5p, miR-30a-5p/miR-324-5p and miR-17-5p/miR-194-5p	95
Glioblastoma	Serum	ExoQuick	TaqMan Human microRNA A Array v2.1 (Applied Biosystems)	RNU48 and median normalization	25 patients with glioblastoma multiforme and 25 healthy controls	miR-483-5p, miR-574-3p, miR-320, miR-197, miR-484, miR-146a, miR-223 and RNU6-1	96
Hepatocellular carcinoma	Serum	Differential centrifugation	3D-Gene Human miRNA Oligo Chip	Subtraction of the background signal mean intensity	4 hepatocellular carcinoma patients with recurrence and 2 without recurrence	Upregulation: miR-1246 Downregulation: miR-718	97
Lung cancer	Plasma	Differential centrifugation	miScript SYBR Green PCR Array (Qiagen, Valencia, CA, USA)	Mean value of miRNA evaluated	30 pooled patients with non-small cell lung cancer and 75 pooled patients without tumor	miR-28, miR-29c, miR-141, miR--144, miR-146, miR-195 and miR-302c	98
Lung cancer	Plasma	ExoQuick	microRNA Ready-to-Use PCR, Human panel I+II, V2.M (Exiqon, Woburn, MA, USA)	let-7a	10 patients with lung adenocarcinoma, 10 with lung granuloma and 10 healthy smokers	miR-502-5p, miR-376a-5p, miR--1974, miR-378a, miR-379, miR--151a-5p, miR-139-5p, miR-200b-5p, miR-190b, miR-30a-3p, miR--629, miR-17, miR-100 and miR-154-3p	73
Lung cancer	Plasma	Size exclusion chromatography and magnetic-activated cell sorting with anti-EpCAM	Custom-developed miRNA arrays covering 467 miRNAs (Invitrogen, Carlsbad, CA, USA)	Spiked-in miRNA	27 patients with lung adenocarcinoma and 9 healthy controls	miR-17-3p, miR-21, miR-106a, miR--146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212 and miR-214	36
Melanoma	Plasma	ExoQuick	nCounter miRNA Expression Assay	Spiked-in miRNA	10 patients with metastatic sporadic melanoma, 8 with familial melanoma, 5 with p16 mutation carrier, 13 healthy controls	miR-17, miR-19a, miR-21, miR-126 and miR-149	84
Ovarian cancer	Serum	Magnetic-activated cell sorting with anti-EpCAM	Custom-developed miRNA arrays covering 467 miRNAs	Spiked-in miRNA	50 patients with ovarian serous papillary adenocarcinoma, 10 with benign ovarian adenoma and 10 healthy controls	miR-21, miR-141, miR-200a, miR--200c, miR-200b, miR-203, miR-205 and miR- 214	37
Pancreatic cancer	Serum	Differential centrifugation and sucrose-gradient centrifugation	miRCURY LNA microRNA array version 7 (Exiqon)	NA	4–5 pooled pancreatic cancer patients and 4–5 pooled healthy controls	miR-1246, miR-4644, miR-3976 and miR-4306	99
Prostate cancer	Plasma	ExoQuick	Small RNA sequencing with HiSeq2000 (Illumina, San Diego, CA, USA)	Read counts per million mappable miRNA sequences	23 patients with castration-resistant prostate cancer	miR-1290, miR-1246 and miR-375	78

Abbreviations: EV, extracellular vesicle; miRNA, microRNA; NA, not applicable.
[a]Indicates miRNAs upregulated in cancer patients, unless otherwise specified.

Table 2 Individual expression analysis of EV-derived miRNAs in cancer

Cancer	*Sample source*	*Isolation method*	*Normalization method*	*Cohort*	*Deferentially expressed miRNAs*[a]	*Ref*
Breast cancer	Serum	ExoQuick	miR-16 and miR-484	50 patients with breast cancer and 12 healthy controls	miR-101 and miR-372	100
Colorectal cancer	Serum	Differential centrifugation	miR-16	209 patients with colorectal cancer and 16 healthy controls	miR-19a	94
Colorectal cancer	Serum	Differential centrifugation	miR-451	13 patients with colorectal cancer and 8 healthy controls	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223 and miR-23a,	83
Esophageal cancer	Serum	ExoQuick	miR-16	51 patients with esophageal squamous cell carcinoma and 41 with benign diseases	miR-21	85
Gastric cancer	Plasma	ExoQuick	Spiked-in miRNA	10 patients with gastric cancer and 10 healthy controls	NA	101
Glioblastoma	Serum	ExoQuick	RUN48	50 patients with glioblastoma multiforme and 30 healthy controls	RNU6-1, miR-320 and miR-574-3p	96
Glioma	Serum	Differential centrifugation	GAPDH	50 patients with glioma and 25 with non-tumor diseases	NA	102
Hepatocellular carcinoma	Serum	ExoQuick	Spiked-in miRNA	20 patients with hepatocellular carcinoma, 20 with chronic hepatitis B and 20 with liver cirrhosis	miR-18a, miR-221, miR-222 and miR-224	103
Hepatocellular carcinoma	Serum	Differential centrifugation	Spiked-in miRNA	59 patients with hepatocellular carcinoma	Downregulation associated with poor prognosis: miR-718	97
Hepatocellular carcinoma	Serum	Total Exosome Isolation Reagent for Serum (Invitrogen)	U6	30 patients with hepatocellular carcinoma, 30 with active chronic hepatitis B and 30 healthy controls	miR-21	86
Laryngeal cancer	Serum	ExoQuick	U6	52 patients with laryngeal squamous cell carcinoma and 49 with polyps of vocal cords	miR-21	87
Lung cancer	Plasma	Differential centrifugation	Mean value of miRNA expression	30 patients with non-small cell lung cancer and 61 patients without tumor	Downregulation: miR-141	98
Lung cancer	Plasma	ExoQuick	let-7a	50 with lung adenocarcinoma, 30 with lung granuloma and 25 healthy smokers	miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100 and miR-154-3p	73
Melanoma	Serum	ExoQuick	miR-16	21 patients with advanced melanoma, 16 disease-free patients with melanoma and 19 healthy controls	Downregulation: miR-125b	104
Ovarian cancer	Serum	Total Exosome Isolation Reagent for Serum	miR-484	163 patients with epithelial ovarian cancer, 20 with benign tumors and 32 healthy controls	miR-373, miR-200a, miR-200b and miR-200c	76
Pancreatic cancer	Serum	Differential centrifugation and sucrose-gradient centrifugation	U43, U6, 18S and 5S rRNA	131 patients with pancreatic cancer, 25 with chronic pancreatitis, 22 with benign pancreatic tumors and 12 with non-pancreatic cancer and 30 healthy controls	miR-1246, miR-4644, miR-3976 and miR-4306	99
Pancreatic cancer	Serum	Differential centrifugation	U6	22 patients with pancreatic cancer, 6 with benign pancreatic tumor, 7 with ampullary carcinoma, 6 with chronic pancreatitis and 8 healthy controls	miR-17-5p and miR-21	88
Prostate cancer	Serum	ExoQuick	Spiked-in miRNA	51 patients with prostate cancer and 40 healthy controls	miR-141	105
Prostate cancer	Serum	Total Exosome Isolation Reagent for Serum	No normalization	8 patients with metastatic prostate cancer, 6 with post-prostatectomy and 10 non-prostate cancer patients	miR-21, miR-375 and miR-574	77
Prostate cancer	Plasma	ExoQuick	miR-30a and miR-30e-5p	100 patients with castration-resistant prostate cancer	Upregulation associated with poor prognosis: miR-1290 and miR-375	78
Uveal melanoma	Serum	Differential centrifugation	U6	12 patients with uveal melanoma and 12 healthy controls	miR-146a	106

Abbreviations: EV, extracellular vesicle; miRNA, microRNA; NA, not applicable.
[a]Indicates miRNAs upregulated in cancer patients, unless otherwise specified.

EVs in serum during clot formation, which may account for over 50% of EVs in serum.[67] However, a review of the literature indicates that two thirds of all circulating EV studies used serum as the EV source (Tables 1 and 2). Serum may be the traditional choice for source material for EV studies, as a significantly higher yield of EVs is obtained from serum.[68]

As indicated previously, no perfect method exists for the identification and purification of exosomes or microvesicles, and current methods are likely to yield diverse populations of EVs. Interestingly, only one third of studies adopted the 'gold standard' method of differential centrifugation for EV isolation. Instead, polymer-based EV precipitation solutions, such as ExoQuick and

Total Exosome Isolation Reagent (Invitrogen, Carlsbad, CA, USA), were widely used, accounting for almost two thirds of these studies. The use of these methods is likely due to their simpler, less time consuming procedures, which might render polymer-based EV isolation methods more feasible options in the clinical setting. However, the purity of the polymer-precipitated EVs is yet to be proven, and these preparations likely do not exclude co-precipitation of other circulating miRNA carriers, such as Ago2 proteins[69] and high-density lipoproteins.[70]

Although only two studies (from the same group) described the use of the immunoaffinity isolation method with magnetic-activated cell sorting, the data produced from these studies are promising.[36,37] Taylor *et al.*[37] isolated EpCAM-positive EVs from patient plasma, and observed that miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214 were all significantly elevated in ovarian cancer patients compared with patients with benign tumors. The authors also showed that the protein yield of EpCAM-positive exosomes was clearly correlated with tumor stage. In cases where tumor-specific EV markers are available, immunoaffinity isolation may be a useful method for isolation of tumor-derived EV miRNAs. For example, glypican-1 (GPC1) was recently identified as a tumor-specific EV surface marker in pancreatic cancer.[71] In a colorectal cancer study, CD147 was found to be an optimal EV surface marker to detect tumor-derived EVs.[72] These specific EV molecular studies yield promising data for the development of an optimal EV isolation method for tumor-specific EVs.

Following EV isolation, miRNA expression may be profiled via quantitative real-time PCR-based arrays, hybridization-based arrays or next generation sequencing (Table 1). Current normalization methods are varied, but can be generally categorized into three types: internal control, spike-in miRNA or global mean normalization. Internal control normalization is a standard method for miRNA expression normalization in tissues. However, it is challenging to identify a reliable internal miRNA/small RNA control for EV studies, due to the limited consensus regarding a consistent miRNA/small RNA in EVs. Cazzoli *et al.*[73] used let-7a as an internal control to normalize lung adenocarcinoma plasma EV miRNA expression profiles, identified following an examination of the expression of five candidate miRNAs in a training cohort. In a hepatocellular carcinoma serum exosome miRNA study, it was shown that a combination of miR-221, miR-191, let-7a, miR-181a and miR-26a was optimal for liver-specific miRNA normalization.[74] Although miR-16, miR-451, miR-484 and U6 are often used as internal controls due to their use in whole plasma or serum studies, it remains unclear whether these miRNAs are suitable for EV miRNA normalization. Externally spiked-in miRNA is frequently used for normalization by adding a set amount of a unique miRNA species (for example, cel-miR-39, cel-miR-54 or ath-miR159a) into the sample prior to RNA isolation. The miRNA expression data derived from samples normalized using spike-in miRNA produces an expression value that is absolute rather than relative; thus the resulting miRNA profile may reflect biological relevance more precisely. However, as this method does not normalize the processes prior to RNA isolation, variation during EV isolation cannot be normalized. The third option for normalization of EV miRNA expression is the global mean normalization method, which uses the mean expression value of all miRNAs as a control, with the assumption that mean expression levels of detectable miRNAs are consistent if input amounts remain constant between samples.[75] The primary advantages of this method are that it controls for external factors that lead to variability in miRNA expression due to sample processing, and it does not rely on a specific miRNA for normalization. The limitation of this method is that it may only be applicable to genome-wide miRNA profiling studies; therefore alternative normalization methods are still required for validation studies.

As a result of the wide-ranging methods for EV isolation, miRNA profiling and normalization, the resulting circulating EV miRNA expression signatures are highly variable between studies, even within a single cancer type (Tables 1 and 2). However, specific miRNAs were consistently deregulated among many of these studies, including the miR-200 family in ovarian cancer,[37,76] and miR-375 in prostate cancer.[77,78] Whole serum (not EV-specific) studies demonstrated that the miR-200 family of miRNAs were upregulated in the serum from ovarian cancer patients, which in turn correlated with increased tumor aggressiveness.[79] Upregulation of miR-375 in the whole serum (not EV-specific) of prostate cancer patients was also frequently reported.[80–82] These studies potentially indicate that the miR-200 family and miR-375 are promising EV-derived biomarkers, warranting further functional validation.

Our review of the literature revealed that miR-21 was upregulated in EVs from different kinds of cancers, including colorectal,[83] lung,[36] melanoma,[84] ovarian,[37] esophageal,[85] hepatocellular carcinoma,[86] laryngeal,[87] pancreatic[88] and prostate[77] cancers (Tables 1 and 2). miR-21 is known to function as an onco-miR, downregulating several tumor suppressor genes such as phosphatase and tensin homolog (PTEN),[89] programmed cell death 4 (PDCD4)[90] and tropomyosin 1 (TPM1).[91] Upregulation of miR-21 in tumor tissue has been shown to be a poor prognostic factor in numerous cancers.[92] Although miR-21 is not cancer-type specific, due to its frequent association with malignancies, this miRNA could be a candidate prognostic biomarker in circulating EVs for certain cancer types.

CONCLUSIONS

EVs are secreted from numerous cell types, including tumor cells, and have been identified as an important mechanism by which cells communicate, via the transfer of DNA, mRNA, miRNA, proteins and lipids. Tumor-derived EVs impact the local and distal environment, aiding in tumor progression, angiogenesis and metastasis. EV-derived miRNAs, which are highly stable in bodily fluids, offer significant promise as circulating biomarkers for assessing tumor aggressiveness, and have been implicated in numerous human malignancies. However, current methods for examining tumor-derived EV miRNA biomarkers are highly variable. A standardized method of EV isolation and miRNA expression assessment and normalization would enable a more reliable inter-study validation of EV miRNAs as biomarkers. Nonetheless, the functional implication of EV-derived miRNAs in cancer, and the ability to detect tumor-derived EV miRNAs in plasma and serum, renders them as highly promising candidates for future application in the clinical setting.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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