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Cell-free microRNAs in blood and other body fluids, as cancer biomarkers

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Abstract

The discovery of cell-free microRNAs (miRNAs) in serum, plasma and other body fluids has yielded an invaluable potential source of non-invasive biomarkers for cancer and other non-malignant diseases. miRNAs in the blood and other body fluids are highly stable in biological samples and are resistant to environmental conditions, such as freezing, thawing or enzymatic degradation, which makes them convenient as potential biomarkers. In addition, they are more easily sampled than tissue miRNAs. Altered levels of cell-free miRNAs have been found in every type of cancer analysed, and increasing evidence indicates that they may participate in carcinogenesis by acting as cell-to-cell signalling molecules. This review summarizes the biological characteristics and mechanisms of release of cell-free miRNAs that make them promising candidates as non-invasive biomarkers of cancer.

Introduction

MicroRNAs (miRNAs) are non-coding RNAs that are ~21 nucleotides in length. They are synthesized in the cell nucleus (1,2), processed by the Drosha enzyme complex (3) and then exported to the cytoplasm by GTP-dependent exportin-5 (4), where they are further processed by the Dicer enzyme complex (5,6) to yield mature miRNAs (7,8). In the cytoplasm, a leader strand of mature miRNA is attached to the RNA-induced silencing complex (RISC) (9,10), and it recognizes messenger RNA (mRNA) with a partially complementary sequence (11), inducing degradation or inhibiting translation of the mRNA (12–15). miRNAs are involved in post-transcriptional gene regulation of virtually all cellular processes that have been investigated so far. These include proliferation, differentiation, apoptosis and haematopoiesis (7,12,16–18). It was soon discovered that cellular miRNA expression levels were altered in multiple pathological conditions, including cancer (12,19–23). In cancer, miRNA expression profiles could distinguish malignant from non-malignant tissue, leading to the search for potential cancer biomarkers. Several studies have suggested that tissue miRNAs could be used as biomarkers for the classification and diagnoses/prognoses of lung cancer, breast cancer, colorectal cancer, pancreatic cancer, hepatocarcinoma and neuroblastoma, among others (24–32). Moreover, experimentally manipulating the levels of specific miRNAs affects many carcinogenic properties of tumour cell lines, such as survival, invasion, metastasis and tumour progression (33–37). Tissue miRNAs have undeniable potential for clinical use, but accessing them in solid tumours remains problematic.

Later, it was discovered that miRNAs were also secreted by a variety of cell types under normal and pathological conditions. These secreted, or cell-free, miRNAs are highly stable and can be delivered to recipient cells in a functional way. It has been hypothesized that secreted miRNAs are mediators of cell-to-cell communication and gene expression, and currently, there is experimental evidence that supports that hypothesis.

Cell-free miRNAs have been found in peripheral blood (referred to as circulating miRNAs) and several other body fluids, such as tears, urine and pleural effusions, exhibiting distinctive expression patterns in healthy individuals (38–40). Similar to tissue miRNAs, altered profiles of cell-free miRNAs were found to be associated with several pathological conditions including cancers (38,41). The accessibility and stability of cell-free miRNAs (38,42,43) makes them valuable non-invasive biomarkers (44), and they have been actively investigated as such.

Cell-free miRNAs in serum and plasma

In 2008, cell-free miRNAs were isolated from the serum and plasma of healthy individuals in a stable form and

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were protected from endogenous ribonuclease (RNase) activity in the blood (38,42).

Mitchell *et al.* (38) first confirmed the presence of small RNA species ranging 10–70 nt in size in human plasma by characterizing the size of total ^{32}P-labelled RNA extracted from healthy donor plasma. Then, they generated a small RNA cDNA library from the 18–24 nt RNA fraction, demonstrating that 91 of 98 sequences corresponded to known miRNAs and directly confirming that mature miRNAs are present in human plasma. Finally, they quantified three known mature miRNAs (miR-15b, miR-16 and miR-24) in the plasma of three healthy donors using specific probes and RT-qPCR. Additionally, Chen *et al.* (42) used semi-quantitative RT-PCR to detect six randomly selected mature miRNAs in human plasma and serum. They then sequenced the PCR products from serum and showed that 87 of 100 sequences corresponded to the appropriate miRNAs. Both studies demonstrated that endogenous miRNAs in serum and plasma are resistant to RNase digestion. Nevertheless, synthetic miRNAs added directly to serum and plasma samples were rapidly degraded, indicating that endogenous miRNAs exist in a protected form that is not related to their intrinsic short length or structure (38,45,46). In contrast, large RNAs from serum were rapidly degraded by ribonuclease digestion, which illustrated the potential biological relevance of cell-free miRNAs in the blood (42). It was later shown that miRNAs are protected by encapsulation in exosomes and by protein-binding complexes (see the *Mechanisms of miRNA release* section). In addition, miRNAs were shown to be resistant to environmental conditions, such as freezing, thawing, pH and enzymatic degradation (38,42), which makes them to be used as potential biomarkers.

In addition to the high stability of cell-free miRNAs, the first studies reported that miRNA levels are consistent among healthy individuals (42,47). Chen *et al.* (42) analysed the levels of 14 randomly selected miRNAs in the serum of seven healthy Chinese individuals (22–25 years old; four men and three women) by reverse transcriptase (RT)-PCR and found that the expression levels were consistent. Gilad *et al.* (47) analysed 18 highly expressed miRNAs in the serum of two unrelated healthy individuals (females; age not specified) by RT-PCR and found that their levels were consistent. Mitchell *et al.* (38) measured the levels of three miRNAs that are found in the plasma at moderate to low levels from three healthy individuals (unspecified age or gender), and they found no differences in expression levels. Unfortunately, none of these studies assessed how individuals were determined to be healthy; only Gilad *et al.* mentioned that they were chosen by self-reporting of good health. On the other hand, it was reported that serum miRNA levels vary with pregnancy in healthy women, suggesting that the levels of miRNAs differ according to physiological status. However, this study analysed pregnant women compared with unrelated non-pregnant women rather than analysing the same subjects when pregnant and when not (47). In addition, it was reported that subjects with diabetes had altered levels of serum miRNAs compared with healthy subjects (42), showing that metabolic diseases could be associated with altered miRNA levels. Nevertheless, there are no studies that systematically analysed the levels of circulating miRNAs from healthy subjects of different ages, ethnicities or physiological states. In addition, the levels of miRNAs during subclinical pathologies or after the natural resolution of infectious diseases have not been investigated. Therefore, miRNA studies should include healthy controls who are matched in age, ethnicity and gender. Equally important, healthy status should be assessed by clinical and laboratory measurements. Additionally, recent reports have suggested that environmental contaminants can alter the expression profile of endogenous miRNAs in human and mouse cells (48–50). There are several studies of endogenous miRNA expression changes attributable to air pollutant exposure in human beings (50–53), but only two studies regarding the effect of environmental contaminants on cell-free miRNAs have been published (54,55). Consequently, studies searching for miRNAs biomarkers may consider the influence of environmental pollution in the alterations of miRNAs levels: for example, people living in highly polluted regions compared with people living in regions without contamination or people exposed to a particular contaminant such as smokers versus non-smokers.

Regarding total concentration of miRNAs from healthy subjects, the cell-free miRNA concentration in serum and plasma is much lower than the miRNA concentration in blood cells from the same individual, although there is an enriched miRNA fraction within the small RNA fraction from serum (42). Moreover, miRNAs comprise >80% of the small RNA fraction from serum compared with <40% of the small RNA fraction from peripheral blood cells (Fig. 1). Consequently, total RNA extraction from the serum would not require the use of techniques to concentrate small RNAs. On the other hand, plasma contains a greater concentration of miRNAs than serum, although it was later demonstrated that this was due mainly to the miRNAs released by platelets during sample processing (56). To accurately analyse cell-free miRNAs in plasma, the sample should be ultra-centrifuged to eliminate platelets before storage (freezing) or RNA extraction (56). Additionally, serum

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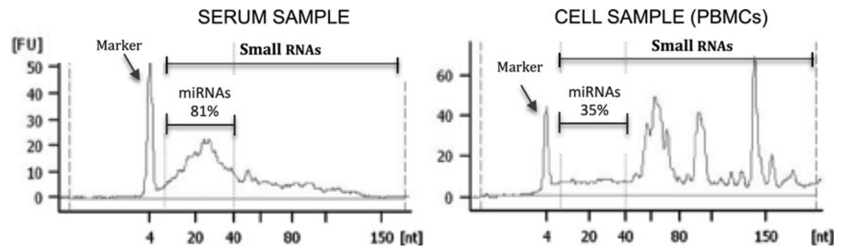

Figure 1. Small RNA profile in serum and peripheral blood mononuclear cells (PBMCs) assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the Small RNA chip.

Table 1. Blood-derived and other body fluids that contain cell-free miRNAs

Body fluid	Healthy subject	Cancer disease	References
Breast milk	Pregnant women	Not reported	39
Colostrum	Pregnant women	Not reported	39
Saliva	Yes	Oral cancer	39,92
Tears	Yes	Not reported	39
Urine	Yes	Bladder and renal cancer	39,40,96,97,99
Seminal fluid	Yes	Not reported	39
Amniotic fluid	Yes	Not reported	39
Pleural fluid	Yes	Not reported	39
Bronchoalveolar lavage	Yes	Lung cancer	39
Pleural effusion	No	Lung and gastric cancer	41,103,104
Gastric juice	Yes	Gastric cancer	91
Pancreatic juice	No	Pancreatic cancer	96
Peritoneal fluid	Yes	Gastric cancer (metastasis)	39,105
Cerebrospinal fluid	Yes	Brain cancer	39,107,108
Plasma	Yes	Lung, breast, prostate, ovarian, bladder, pancreatic, gastric, liver, colorectal, oral and oesophageal cancer	27,74,75,77–81,83,84,86,88
Serum	Yes	Lung, breast, prostate, ovarian, gastric, liver and oesophageal cancer	59–63,65–69,78

obtained from haemolysed samples contains increased concentrations of certain miRNAs due to release from erythrocytes. Mature erythrocytes contain abundant miRNAs even when they lack ribosomal and large RNAs (57,58). According to McDonald *et al.* (56), the levels of four miRNAs were not altered if serum samples contained <10 mg/dl of haemoglobin; therefore, this parameter could be used for the quality control of samples.

Early studies of circulating miRNAs revealed that while the levels of serum miRNAs in healthy individuals remained constant, the levels of miRNAs in patients with prostate cancer (38), lung cancer and colorectal cancer (42) were altered and distinguishable from healthy individuals. Moreover, it was shown that miRNAs originating from human prostate cancer cells were detected in the plasma of NOD/SCID mice after xenograft transplantation (38). All of this evidence prompted a search for circulating miRNAs in serum and plasma that could be potential non-invasive biomarkers for cancer. Biomarkers that can be noninvasively sampled are particularly important for solid tumours because tissue samples for histopathological evaluation require invasive and sometimes dangerous procedures.

Altered levels of miRNAs have been found in serum and plasma from patients with every type of solid tumour analysed thus far, and miRNA signatures have been reported to be potentially useful for diagnoses and prognoses of lung, breast, prostate, ovarian, bladder, pancreatic, gastric, liver, colorectal, oral and oesophageal cancer (27,38,42,59–89) (Table 1). Table 2 summarizes the candidate cell-free miRNA tumour biomarkers that have been reported in the literature during the last 5 years.

Cell-free miRNAs in other body fluids

To date, cell-free miRNAs have been detected in every body fluid analysed. Serum, plasma, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid, seminal fluid, saliva and gastric juices from healthy individuals all contain miRNAs (Table 1) (39,40,90–92). They have

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Table 2. Cell-free microRNAs detected in serum and plasma as potential biomarkers for cancer

Blood-derived fluid	Cancer disease	Study design	Differentially expressed miRNAs	Method	References
Serum	Lung cancer	NSCLC versus healthy controls (HC)	10 miRNA signature: miR-20a, miR-24, miR-25, miR-145, miR-152, miR-199a-5p, miR-221, miR-222, miR-223, miR-320	RT-qPCR	59
		NSCLC-asymptomatic high-risk individuals versus healthy smokers	34-miRNA signature	RT-qPCR	60
		NSCLC longer versus NSCLC shorter survival	miR-486, miR-30d, miR-1 and miR-499; overall survival	Solexa sequencing and RT-qPCR	62
		NSCLC versus healthy controls (HC)	miR-21	MicroArray and RT-qPCR	61
		NSCLC versus healthy controls (HC)	miR-21	RT-qPCR	65
		NSCLC versus healthy controls (HC)	miR-15b and miR-27b	RT-qPCR	66
	Breast cancer (BC)	Primary BC after surgery (M0), metastatic BC (M1) versus HC	miR-17, miR-34a, miR-155 and miR-373	RT-qPCR	69
		Primary BC (M0) versus metastatic BC (M1) versus HC	miR-155 (M0 from HC), miR10b-34a & -155 (M1 from HC and metastases)	RT-qPCR	68
		BC versus HC	miR-195	RT-qPCR	67
		BC versus HC	miR-21, miR-106a and miR-155	RT-qPCR	63
	Prostate cancer (PC)	Metastatic PC versus non-recurrent PC	miR-141 and miR-375 (metastatic PC)	RT-qPCR	78
	Gastric cancer (GC)	GC versus HC	miR-1, miR-20a, miR-27a, miR-34 and miR-423-5p	Solexa sequencing and RT-qPCR	64
	Oesophageal cancer (ESCC)	ESCC versus HC	miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a and miR-127-3p	Solexa sequencing and RT-qPCR	73
Plasma	Lung cancer	NSCLC versus COPD versus HC	Signature (high miR-155-5p, high miR-223-3p, and low miR-126-3p) progression AD Signature (high miR-20a-5p, low miR-152-3p, and low miR-199a-5p) survival SCC	RT-qPCR	74
		NSCLC versus HC	let-7f, miR-20b and miR-30e-3p (NSCLC) let-7f and -30e-3p (poor survival)	RT-qPCR	233
		NSCLC versus before and at the time of disease versus HC	miR-17, miR-660, miR-92a, miR-106a, and miR-19b (diagnosis) miR-660, miR-140-5p, miR-451, miR-28-3p, miR-30c and miR-92a (predictive) mir-221, miR-660, miR-486-5p, miR-28-3p, miR-197, miR-106a, miR-451, miR-140-5p and miR-16 (predictive aggressive cancer)	RT-qPCR	27
		NSCLC versus HC	miRNA-21, miRNA-126, miRNA-210, and miRNA-486-5p	RT-qPCR	75
	Breast cancer (BC)	BC early-stage Caucasian (CA), African-American (AA) versus HC	let-7c, miR-589 (CA); miR-425* and let-7d* (AA)	MicroArray and RT-qPCR	77

(continued)

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Table 2. (continued)

Blood-derived fluid	Cancer disease	Study design	Differentially expressed miRNAs	Method	References
	Prostate cancer (PC)	PC versus HC	miR-107 and miR-574-3p (PC versus HC) miR-141, miR-375 and miR-200b (metastatic versus non-metastatic)	RT-qPCR	78
		PC stage 2 and 3	miR-20a (stage 3); miR-20a and miR-21 (high-risk CAPRA score)	RT-qPCR	79
	Ovarian cancer (OC)	Endometriosis (Ea) versus Ea-associated OC (EAOC) versus HC versus and serous ovarian cancer (SOC)	miR-16, miR-21 and miR-191 (EAOC versus HC) miR-21, miR-362-5p and miR-127a (Ea versus EAOC) miR-16, miR-191 and miR-4284 (Hc versus SOC)	RT-qPCR	80
	Bladder cancer (BdC)	Muscle invasive BdC (MIBdC) versus non-muscle-invasive BdC (NMIBdC) versus noncancerous controls	miR-200b (MIBdC)	MicroArray	81
	Pancreatic cancer (Pc)	Pc versus HC	miR-210	RT-qPCR	82
	Gastric cancer (GC)	GC versus HC	miR-17-5p, miR-21, miR-106a, miR-106b and let-7a	RT-qPCR	83
	Liver cancer (LC)	LC versus HC	miR-483-5p	RT-qPCR	234
		HBV-related LC versus HC versus hepatitis B versus cirrhosis	miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801 (HBV-related LC)	MicroArray and RT-qPCR	84
	Colorectal cancer (CRC)	CRC versus HC	miR-601 and miR-760	PCR array and RT-qPCR	103
		Advanced CRC versus HC	miR-29a and miR-92a	RT-qPCR	87
	Oesophageal cancer (ESCC)	ESCC versus HC	miR-16, miR-21, miR-185 and miR-375 (ESCC) miR-16 (T3-4 versus T1-2) miR-16 and miR-21 (survival)	RT-qPCR	86
		ESCC (3-year follow-up)	miR-21 and miR-375 (shorter survival)	RT-qPCR	88

NSCLC, non–small-cell lung cancer; ESCC, Oesophageal squamous cell carcinoma; COPD, chronic obstructive pulmonary disease; AD, lung adenocarcinoma; SCC, squamous cell carcinoma; CAPRA, Cancer of the Prostate Risk Assessment score; T, stages of disease.

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also been detected in pancreatic cyst fluids (93). Sadakari *et al.* (94) reported the presence of miRNAs in pancreatic fluid, but the total RNA was extracted from a cell pellet; therefore, they were not cell-free or secreted miRNAs. It was reported that cell-free miRNAs showed a distinctive expression pattern in each body fluid analysed (39,95), suggesting a potential biological function associated with the surrounding tissues. These distinctive expression patterns include variations in miRNA concentrations in different body fluids, as well as differences in the species of miRNAs detected. For example, of 12 body fluids analysed, saliva, breast milk and seminal fluid had the highest number of detectable miRNA species (~400), whereas urine, cerebrospinal fluid, and pleural fluid had the fewest detectable miRNA species (~200) (39). Weber *et al.* (39) also reported variation in the concentration of total RNA obtained from 300 μl of different body fluids of 113–48 240 μg/l. In addition, it was reported that highly abundant miRNAs are detected in many different fluids, but several miRNAs are present exclusively in one body fluid, such as miR-637 in tears and miR-193b in breast milk (39). According to Hanson *et al.* (95), a panel of nine miRNAs allows for the identification of the body fluid of origin of forensic samples. In addition, it was shown that cell-free miRNAs in urine could be used to detect physiological status such as pregnancy in normal individuals (39,47).

Similar to cell-free miRNAs in serum and plasma, there is evidence to indicate that alterations in the levels of cell-free miRNAs in other body fluids might indicate diseases such as oral (92), bladder (40,96–98), renal (99), lung (41,100–104), gastric (91,105), pancreatic (106) and brain cancer (107,108) (Tables 1 and 3). In particular, saliva and urine are fluids that are easy to obtain and do not require invasive procedures. Table 3 summarizes the miRNAs that are candidates as biomarkers for cancer that have been reported in the literature to date.

Mechanisms of miRNA release

The potential usefulness of circulating miRNAs as reliable biomarkers in cancer may be based on their potential biological function. If cells actively secrete miRNAs as a mechanism of cell communication, then the levels and patterns of these miRNAs should be specific and would depend on the type of secretory cell and its metabolic activity. Because cancerous cells have higher metabolic activity than normal cells, it is feasible that certain miRNAs released from those cancerous cells can be used as biomarkers of cancer. Therefore, it is necessary to understand the release mechanisms of miRNAs.

Experimental evidence indicates that four potential forms of miRNAs are released from cells: miRNAs that are encapsulated within exosomes (109), complexed with Argonaute2 protein (Ago2) (45,46), bound to high-density lipoprotein (HDL) (110) or bound to the RNA-binding protein nucleophosmin (NPM1) (111) (Fig. 2). Importantly, none of this evidence contradicts the existence of one or another extracellular form of miRNA in any cell line or biological sample analysed. In addition, it has been suggested that miRNAs are released from broken cells passively, due to injured tissue, chronic inflammation, apoptosis or necrosis (42,46). These processes regularly occur in cancer, and it has been suggested that the high stability of these leaked miRNAs could be associated with their binding to Ago proteins (46). However, a systematic or quantitative analysis of the passive release of miRNAs has not been reported.

Apart from the leaked free miRNAs, it has been hypothesized that miRNAs are secreted as mediators of cell-to-cell communication and gene regulation. Consistent with this hypothesis, it has been shown that secretion of miRNAs *via* exosomes and HDL is energy dependent, as they are transported and delivered to other cells in a functional state (109,110,112–115). There is still no direct evidence that miRNAs bound to AGO2 and NPM1 are actively released from cells or that they are transported to other cells.

Exosomes

In 2007, Valadi *et al.* (109) provided the first evidence that exosomes from human and mouse mast cell lines contained miRNAs that were transferred to other cells and maintained their functional capacity. Soon, it was shown that both normal and tumour cells secreted miRNAs contained in exosomes (116–119). Moreover, evidence suggested that tumour cells are capable of influencing their microenvironment and promoting cancer development through exosomal miRNAs (114,116,120).

Exosomes are homogenous membrane vesicles of endocytic origin (20–90 nm) that are released into the extracellular medium by the merging of multivesicular bodies (MVB) with the plasma membrane using active secretion (121,122). First, early endosomes are formed by the fusion of endocytic vesicles from the plasma membrane. These endosomes mature to late endosomes where intraluminal vesicles bud off into the lumen to form MVBs. MVBs directly fuse with the plasma membrane and release exosomes into the extracellular medium. These exosomes contain small RNAs, cytoplasmic proteins and cell receptors, which can be transferred to recipient cells (116,118,123) (Fig. 2). Exosome

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Table 3. Cell-free microRNAs detected in other body fluids as potential biomarkers for cancer

Body fluid	Cancer disease	Study design	Differentially expressed miRNAs	Method	Number of miRNAs analysed	Number of samples	References
Saliva	Oral cancer (OSCC)	OSCC versus healthy controls (HC)	miR-125a and miR-200a ↓	RT-qPCR	314	50 OSCC, 50 HC	92
Urine	Bladder cancer (UCC)	Invasive UCC versus superficial UCC versus HC	Invasive UCC: miR-618 and miR-1255b-5p ↑	MicroArray RT-qPCR	754 6	4 versus 4 versus 4 19 versus 16 versus 20	96
	Bladder cancer (UCC)	UCC versus controls*	miR-1224-3p and miR-15b↑ mR-135b↓	RT-qPCR	15	68 UCC, 53 controls	97
	Renal cancer (RCC)	RCC & benign cocytoma (Bco) versus controls **	miR-15a ↑ (RCC)	RT-qPCR	1	18 RCC, 5 Bco and 5 controls	99
	Bladder cancer (UCC)	UCC versus HC versus urinary tract infection (UTI)	miR-96 and miR-183 ↑ (UCC versus HC only)	RT-qPCR	3	100 UCC, 45 HC, 25 UTI	98
	Bladder cancer (UCC)	UCC hi, UCC lo versus HC versus UTI	miR-126 and miR-182 ↑ (UCC)	RT-qPCR RT-qPCR	157 3	9 each 47 each	40
Bronchoalveolar lavage	Lung cancer (LC*)	LC versus benign lung diseases (BL*)	miR-1285, miR-1303, miR-29a-5p, 650 ↑	PCR-Array RT-qPCR	700 8	3 pools LC and BL (10 each) 30 LC, 30 BL	100
	Lung cancer (NSCLC)	NSCLC versus cancer-free controls	miR-21, miR-143, miR-155, miR-210 and miR-372	RT-qPCR	5	21 NSCLC, 10 controls	101
Pleural effusion	Lung cancer (AD)	AD versus benign pleural effusion (BPE)	miR-134, miR-185, miR-22↓	RT-qPCR	3	45 AD, 42 BPE	102
	Lung cancer (AD)	AD versus BPE*	miR-198 ↓	MicroArray RT-qPCR	160 1	10 AD, 10 BPE 42 AD4, 5 BPE	41
	Lung cancer (NSCLC)	NSCLC longer survival (LS) versus NSCLC shorter survival (SS)	miR-93, miR-100, miR-134, miR-151, miR-345 (survival indicators)	MicroArray & RT-qPCR	Not specified 5	10 (5LS, 5SS) 184 (92LS, 92SS)	103
	Lung + gastric cancer (malignant)	Malignant (Ma) versus benign(Be)	miR-24, miR-26a and miR-30d ↑	RT-qPCR	22 3	Pools 18 Ma and 12 Be 18 Ma versus 11 Be	104
Gastric juice	Gastric cancer (GC)	GC versus gastric ulcer versus normal mucosa/ gastritis	miR-21 and miR-106a ↓	RT-qPCR	2	42 GC, 34 ulcer, 18 normal	91
Pancreatic juice	Pancreatic cancer (PDAC)	PDAC versus NPNH and chronic pancreatitis (CP)	miR-205, miR-210, miR-492 and miR-1247↑ (PDAC)	MicroArrays RT-qPCR	470 4	6 PDAC, 2 pools 6 NPNH each 44 PDAC, 19 CP and 13 NPNH	106
Peritoneal fluid	Metastasis of GC	GC stage T4 versus GC T1-T3 versus GC malignant ascites (MA) versus supernatant metastatic cell lines (Sn)	miR-21, miR-1225-5p↑ (serosa-invasive GC)	MicroArray	1126	3 GCT4, 3 GCT1-3, 4MA, 2 Sn	105
Cerebrospinal fluid	Brain cancer (glioblastoma)	Glioblastoma versus brain trauma non-tumour control	miR-21↑ (recurrence and poor prognosis)	RT-qPCR	1	70 glioblastoma, 25 controls	107
	Brain cancer (Glioblastoma)	Glioblastoma versus non-tumour control * (Ctl)	miR-21↑	RT-qPCR	1	13 glioblastoma, 14 Ctl. 2 ns set: 15 glioblastoma, 15 Ctl	108

(continued)

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Table 3. (continued)

Body fluid	Cancer disease	Study design	Differentially expressed miRNAs	Method	Number of miRNAs analysed	Number of samples	References
Cyst fluid	Pancreatic duct neoplasm (IPMN)	Cystic lesions: IPMNs hi versus IPMNs lo versus SCAs	miR-24, miR-30a-3p, miR-18a, miR-92a, miR-342-3p, miR-99b, miR-106b, miR-142-3p, miR-532-3p, miR-21	PCR-Array RT-qPCR	377 18	15 (5 versus 5 versus 5) 50 (6 versus 14 versus 20)	93

LC*, mix SCC & AD; SCC, squamous carcinoma; AD, lung cancer adenocarcinoma; NSCLC, non–small-cell lung cancer; BL*, fibrosis, pneumonia, chronic obstructive pulmonary disease or tracheal stenosis; BPE, benign pleural effusion (tuberculosis, pneumonia and transudate); BPE*, benign pleural effusion (tuberculous pleurisy or parapneumonic effusion); NSCLC, non–small-cell lung cancer; Be, benign (pleural effusions from patients diagnosed with liver cirrhosis, tuberculosis, pneumonia, heart failure or injury); HC, healthy controls; OSCC, oral squamous cell carcinoma; UCC, urothelial cell carcinoma; UCC hi & lo, high and low grade; controls*, treated for benign urinary conditions and without urinary pathology; RCC, malignant renal cell carcinoma; controls **, urinary inflammatory conditions; PDA, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary mucinous neoplasms; non-tumour control *, head trauma, subarachnoid haemorrhage, normal pressure hydrocephalus, arteriovenous malformation; IPMN hi & lo, high-grade & low-grade IPMNs; SCAs, serous cystadenomas; PDAC, pancreatic ductal adenocarcinoma; NPNH, no pancreatic disease, non-healthy controls; ↓, downregulated; ↑, upregulated.

biogenesis requires the endosomal sorting complex required for transport (ESCRT complex) (124) as well as associated proteins, such as Alix (125) and Tsg 101 (126). Evidence suggests that GW182 and AGO2, which are two main components of the RISC, are enriched in MVBs and could be involved in the function of miRNAs (127,128) (Fig. 2). Li *et al.* (128) reported evidence that AGO2 selectively protects the miRNAs in exosomes, providing a second model of stability of circulating miRNAs in addition to the exosomal lipid bilayer. Recently, Lv *et al.* (129) showed that AGO2 facilitates the packaging of miR-16 into MVBs secreted by HeLa cells. In addition, they reported that incubation of 293T cells with MVBs from HeLa cells co-transfected with HA-tagged-AGO2 and miR-16 induced a higher inhibition of Bcl2 (the putative target of miR-16) expression in recipient 293T cells than incubation with MVBs containing only miR-16. On the other hand, Villarroya-Beltru *et al.* (130) showed that mature miRNAs contain specific motifs that are recognized by the sumoylated-heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), which controls their loading into MVBs. Likely, the mature miRNAs along with AGO2-GW182 and hnRNPA2B1 are loaded into MVBs and released as exosomes into the extracellular medium. Although the mechanisms of exosome interactions with the receptor cells are not well known, labelling of purified exosomes with the green fluorescent lipid dye PKH67 showed that exosomes carrying miRNAs fuse with the plasma membrane of the target cells (113,131).

Ago2-associated miRNAs

The first studies that explored the possibility of extracellular miRNAs outside exosomes in clinical samples were reported in 2011 (45,46). Turchinovich *et al.* (46) reported that of 188 miRNA species detected in plasma, 97 miRNAs were detected in both exosomal and exosome-free fractions (51%), while 69 were found exclusively in the exosome-free fraction (37%) and 22 were detected exclusively in exosomes (11%). Arroyo *et al.* (45) reported that of 128 miRNAs detected in plasma, 66% were detected in the exosome-free fraction and 43% were detected only in this fraction. In addition to showing the presence of exosome-free miRNAs, these studies revealed that specific miRNAs were exclusively detected in one or another fraction, suggesting the existence of selective release mechanisms. More importantly, using differential centrifugation purification and immunoprecipitation methods, they showed that exosome-free miRNAs were associated with Ago2, a major component of the RISC (9) (Fig. 2), and this association protects miRNAs from RNase A activity. This finding

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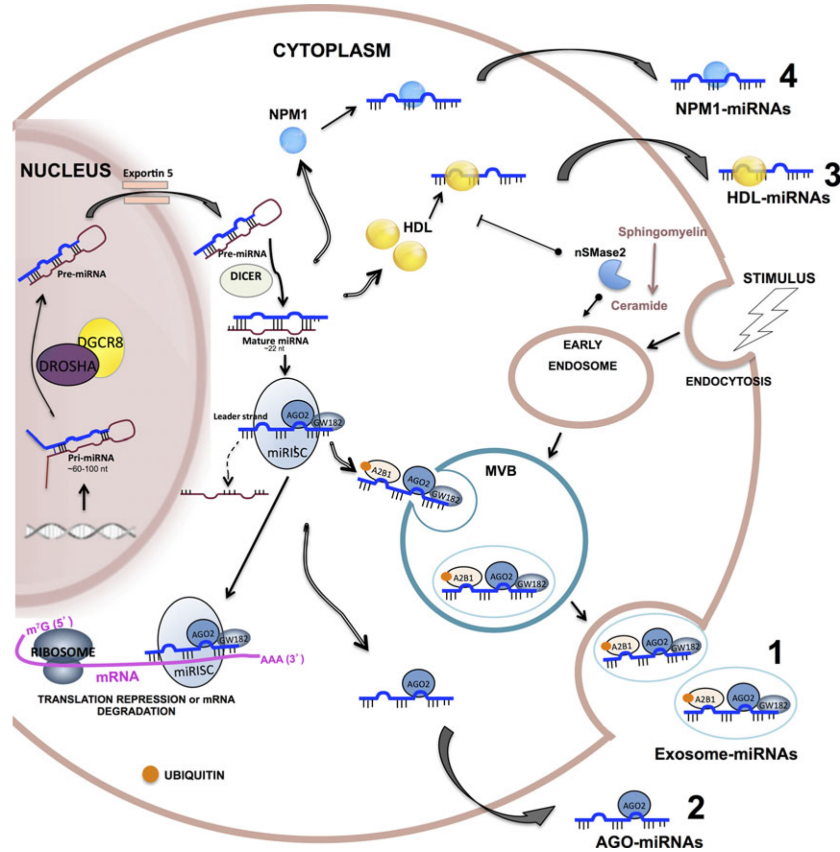

Figure 2. Biogenesis and proposed release mechanisms of free-cell miRNAs. miRNA precursors (pri-miRNAs) are transcribed in the nucleus and processed by the Drosha complex to generate pre-miRNAs. Pre-miRNAs are exported to the cytoplasm *via* exportin-5 and are excised by DICER into a mature form of double-stranded RNAs that are ~22 nucleotides long. One strand is loaded into the RISC along with Argonaut 2 (AGO2) and GW182 to form the miRISC. There, miRNAs can bind to complementary sequences on target mRNAs to repress translation or trigger mRNA cleavage. miRNAs can also be transported to the extracellular environment *via* four proposed mechanisms. (1) Encapsulated within exosomes: miRNAs are sorted into multivesicular bodies (MVBs), which are derived from early endosomes, through a mechanism that requires ceramide production on the cytosolic side by neutral sphingomyelinase 2 (nSMase2), the endosomal sorting complex required for transport (ESCRT) machinery and the sumoylated hnRNPA2B1 protein, which specifically binds mature miRNAs and controls their loading into MVBs. MVBs fuse with the plasma membrane and release exosomes into the extracellular medium. MVBs are enriched with GW182 and AGO2, the two main components of the RISC, which could be involved in the function of miRNAs. (2) Complexed with Argonaute2 protein (Ago2): miRNAs can be stably exported when they are associated with Ago2, a major component of the RISC. (3) Bound to high-density lipoprotein (HDL): miRNA can also be stably exported in conjunction with HDL, *via* a mechanism that is repressed by nSMase2. (4) Bound to the RNA-binding protein nucleophosmin (NPM1): NPM1 was shown to bind and protect exosome-free miRNAs from RNase activity in blood circulation.

revealed an alternative mechanism of miRNA release and cell communication. However, there is no direct evidence that cells actively secrete miRNAs associated with Ago2, that they are taken up by recipient cells, or that there are specific Ago2-miRNA complexes associated with cancer.

NPM1-associated miRNAs

Wang *et al.* (111) first reported that cell-free miRNAs were found in both exosomes and exosome-free fractions obtained from the supernatants of tumour cell lines cultured after serum deprivation. Moreover, similar to the studies by Turchinovich *et al.* (46) and Arroyo *et al.* (45), they found that there are miRNA species exclusively in one or another fraction. These authors found important levels of the RNA-binding protein nucleophosmin 1 (NPM1) in the exosome-free fraction from fibroblast culture supernatants (Fig. 2), and they showed that NPM1 binds and protects miRNAs from RNase activity. They did not report the discovery of Ago2 in their study. However, this study did not provide direct evidence that NPM1-associated miRNAs are taken up by recipient cells, that they are secreted *via* an energy-dependent mechanism or that they are detected in clinical samples.

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HDL-associated miRNAs

High-density lipoprotein-associated miRNAs were found in plasma from healthy human beings and from patients with familial atherosclerosis, stable coronary artery disease and acute coronary syndrome (110,132). Notably, it was reported that the HDL-miRNA profile was distinctly different than the exosome-miRNA profile from matched healthy individuals, which correlates with previously described data suggesting that more than one selective mechanism for miRNA release exists (110). Vickers *et al.* (110) also reported that HDL purified from human plasma transfers miRNAs into cultured hepatocytes (Huh7) with functional capacity, while Tabet *et al.* (115) showed that HDL suppresses the expression of intercellular adhesion molecule-1 (ICAM-1) through the transfer of miR-223 to endothelial cells (HCAEC).

The exact mechanism by which HDL is loaded with miRNAs and which proteins could facilitate this association are not known. However, it was hypothesized that HDL could bind to extracellular plasma miRNAs through divalent cation bridging (110). Vickers *et al.* reported that the inhibition of nSMAse2 significantly increased the amount of miR-223 exported to HDL using J774 macrophages, suggesting that nSMase2 and the ceramide pathway repress miRNA export to HDL (Fig. 2). Conversely, it was previously reported that overexpression of nSMase2 and activation of the ceramide pathway induced export of miRNAs by exosomes (112).

Distinct HDL-miRNA plasma profiles were found in familial hypercholesterolaemia (FH) patients compared with healthy individuals (110), but none have been published so far regarding cancer.

Circulating and body-fluid miRNAs as biomarkers in cancer

A biomarker is defined as any cellular, molecular or genetic component that can be measured and associated with a biological process, pathogenic process or pharmacologic response to treatment (44). Current studies aiming to identify circulating miRNAs with diagnostic value in cancer are mainly based on the different expression profiles of miRNAs in samples from patients with cancer compared with healthy individuals, as the first approach in the pre-clinical exploratory phase. These differentially expressed miRNAs should be able to distinguish cancer patients from non-cancer subjects, which requires measuring the sensitivity and specificity of the candidate biomarker in a second exploratory phase (133), preferably in an independent cohort. Finally, a biomarker for cancer diagnosis should be able to detect a specific type of cancer in a general population with a high specificity and sensitivity. Evidence has indicated that circulating and body-fluid miRNAs are potential biomarkers for diagnosis, as well as biomarkers for prognosis and indicators of disease stage in several cancers.

Nevertheless, a suitable cancer biomarker has to be associated with the presence of tumour cells or the malignant process; therefore, the hypothesis that tumour cells are the main source of the secreted miRNAs with altered levels in cancer patients is relevant. Identifying actual cancer-related miRNAs is crucial, considering that the levels of circulating miRNAs seem to depend on several normal and pathological physiological conditions, such as pregnancy, diabetes and hypertension (42,47,134–136).

We summarized two types of evidence that may indicate that certain upregulated or downregulated miRNAs in cancer patients are associated with malignant processes and, potentially, cancer diagnosis.

Before and after tumour removal

There are studies that have reported that highly expressed circulating miRNAs from cancer patients return to normal levels after tumour resection, suggesting that such miRNAs are of tumour origin. First, several studies designed screening strategies to identify candidate miRNAs with diagnostic value by comparing the serum or plasma of cancer patients to that of healthy controls. Then, they analysed the levels of the candidate miRNAs in pre- and post-surgery samples from patients diagnosed with breast cancer, osteosarcoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, tongue squamous cell carcinoma and gastric cancer (67,83,137–141).

In contrast, Konishi *et al.* (142) first used microarray analysis to compare the expression levels of miRNAs in pre- and post-operative paired plasma samples from gastric patients. They then confirmed the levels of nine candidate miRNAs, which were markedly decreased in the post-operative plasma, in a large cohort using RT-qPCR. Finally, they compared the miRNA levels of two candidate miRNAs between 56 cancer patients and 30 healthy controls and found that they were significantly decreased in post-operative plasma in 90 and 93% of patients (miR-451 and miR-486, respectively). Correspondingly, this strategy was used to identify serum and plasma miRNAs with diagnostic value in colorectal cancer, cervical squamous carcinoma and lung carcinoma (143–145). In addition, Li *et al.* (146) used this strategy to identify serum miRNAs to predict post-operative disease recurrence for stage II/III colorectal cancer patients.

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Similar to the findings in serum and plasma, the expression levels of two upregulated candidate miRNAs in the urine of urothelial carcinoma patients (miR-96 and miR-183) were significantly lower in urine collected after surgery from the same patients (98), suggesting tumour origin of such altered miRNAs.

Role of secreted miRNAs in oncogenesis

In cancer, tumour cells may deliver specific miRNAs to their surroundings to promote tumour survival. Those miRNAs could be associated with potential carcinogenic mechanisms that may overcome the normal cellular environment, making such miRNAs suitable biomarkers for the detection of tumour cells.

Correspondingly, the analysis of the potential roles of candidate miRNAs in oncogenesis is a good supportive approach for choosing reliable miRNAs with diagnostic potential during the exploratory phase of biomarker discovery.

First, the function of a miRNA depends on its target gene; therefore, a miRNA could function as either a tumour suppressor or an oncogene during cancer development. Accordingly, miRNAs functioning as oncogenes (also named oncomirs) promote tumour development by inhibiting tumour suppressor genes, mainly when they are upregulated. miRNAs functioning as tumour suppressors prevent tumour development by negatively inhibiting oncogenes or genes that control cell differentiation or apoptosis; however, downregulation of these miRNAs could lead to cancer development.

For example, miR-10b is highly expressed in the metastatic breast cancer cell line MDA-MB-231 compared with the non-metastatic breast cancer cell line HMLE or non-malignant breast cells (147). This miRNA was also actively secreted *via* exosomes, and it was shown that treatment with miR-10b-enriched exosomes suppressed protein levels of its target genes HOXD10 and KLF4 in HMLE target cells. As a result of this suppression, HMLE cells acquired invasion ability (147). Other studies have also reported that low expression of HOXD10 and KLF4 induces cell migration and metastasis (148,149); therefore, miR-10b, which is found to be upregulated in breast and bladder cancer (147,148,150,151), could be considered an oncogene.

Another example is miR-105 (152), which is expressed and secreted by highly metastatic breast cancer cells (MBC) isolated from pleural effusions. MBC-secreted miR-105 can be transferred to endothelial cells *via* exosomes and results in a significant decrease in the expression of its putative target gene *zonula occludens* (ZO-1), which induces the downregulation of tight junctions and the destruction of the barrier function of endothelial monolayers. Transfecting the recipient cells with a miR-105 inhibitor abolished this effect. It was also demonstrated that cancer-secreted miR-105 induces vascular permeability and promotes metastasis *in vivo*.

The well-known oncomir miR-21 is highly expressed in several tumours, including breast, ovarian, colorectal, lung and leukaemia (153). miR-21 targets tumour suppressors, such as PTEN (154) and TPM1 (155).

An example of a miRNA functioning as a tumour suppressor is miR-152. miR-152 has been found to be downregulated in glioblastoma stem cells (156), non–small-cell lung cancer (NSCLC) tissues (157,158), gastric cancer tissues (159) and hepatocellular carcinoma cells (160). All of these studies reported that overexpression of miR-152 reduced the proliferation, migration and invasion capacity of tumour cells. Although the authors reported different target genes in every study, the expression level of each target gene was associated with those common cellular functions. In addition, evidence indicates that the function of a miRNA depends on the type of cell that the miRNA acts on. miRNAs such as miR-125b, miR-29 and miR-146 have been described as oncogenes or suppressors in different cell types (161–165). Consequently, identifying the potential roles of candidate miRNAs in oncogenesis may require the analysis of the specific expression/release patterns and the identification of specific target genes in each type of tumour cell.

Role of secreted miRNAs in cancer progression: metastasis

Cancer progression involves the regulation of the cellular and tissue microenvironment to promote carcinogenesis and spreading of cancerous cells to distant anatomic sites. This regulation involves cell–cell contact-mediated signals and cell-to-cell signals mediated by secreted factors. miRNAs, in addition to functioning as oncogenes and oncosuppressors inside cancer and stromal cells, are secreted and taken up by nearby cells within the cancer microenvironment and by distant cells in other tissues or organs, thereby delivering regulatory signals that potentially participate in the spreading of those cancer cells. This section describes recent experimental evidence regarding the role of miRNAs in cancer progression, focusing on pivotal mechanisms involved in metastasis and on the secreted form of miRNAs.

Metastasis and epithelial-mesenchymal transition

Metastasis is a multistep biological process that involves dissemination of cancer cells to surrounding and distant organs sites for the formation of new tumour lesions.

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This process is frequently referred to as the invasion-metastasis cascade (166). During metastasis, tumour cells exit their primary sites of growth by breaching the basement membrane, intravasate into the blood and lymphatic vessels, disseminate through the lymphatic system and blood circulation, extravasate to nearby and distant organs and finally adapt to a new microenvironment for metastatic colonization (167). Metastasis, not the primary tumour, is the major cause of mortality in cancer patients, and it plays a critical role in tumour recurrence and poor prognosis; therefore, it is clinically relevant (168).

In carcinomas arising from epithelial tissues, the epithelial–mesenchymal transition (EMT) is an early step of cancer metastasis, which is characterized by the breaching of the basement membrane that separates epithelial cells from multiple layers of stroma. The EMT is a highly conserved process characterized by a transition from immotile epithelial cells to motile mesenchymal cells. The EMT is fundamental for normal embryonic development; however, it is aberrantly activated during cancer progression, providing cancer cells the ability to migrate and form distant metastases (169,170). This transition process is characterized by a decrease in the expression of cell–cell contact proteins such as the epithelial marker E-cadherin and by the loss of cell–cell junctions and apico-basal cell polarity (169–171).

Several endogenous miRNAs associated with metastasis have been identified, and their roles in the EMT have been reported recently (172,173). However, there is limited experimental evidence regarding the participation of the secreted forms of such miRNAs in metastasis-EMT. Still, several circulating miRNAs have been identified as biomarkers for cancer metastasis.

Toiyama *et al.* (174) specifically analysed the diagnostic value of miR-200 family members that were previously associated with the regulation of the EMT in cancer cells in the serum of colorectal cancer patients. They showed that high expression of serum miR-200c was significantly associated with a metastatic phenotype, lymph node metastasis, liver metastasis and the development of distant metastasis in colorectal cancer patients. They analysed the levels of four candidate miRNAs (miR-200b, miR-200c, miR-141 and miR-429) in the serum samples of stage I ($n = 12$) and stage IV ($n = 12$) patients and further validated the increased levels of miR-200c in stage IV patients in a larger independent cohort of 182 patients and 24 normal controls. The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) was the first group of endogenous miRNAs reported to regulate the EMT, and their roles in tumour progression have been associated with several cancers (175–178). They inhibit the EMT by retaining the epithelial phenotype by targeting the E-cadherin transcriptional repressors ZEB1 and ZEB2, which results in upregulation of E-cadherin (179,180). In this study, the authors also reported that miR-200c was overexpressed in liver metastases compared with matched primary colorectal cancer tissues, which was in agreement with a previous study in which they demonstrated that miR-200c was overexpressed in metastatic colorectal cancer tissue compared with matched primary colorectal cancer tissue (181). The authors speculated that the origin of serum miR-200c could be from the metastatic site, but no further studies regarding the potential function of this circulating miRNA were performed. One hypothesis is that the low expression of miR-200c in primary tumours facilitates the EMT and exit of cancer cells, but, after metastasis, high expression inhibits the EMT and facilitates the settlement and proliferation of cancer cells, which undergo the opposite process known as the mesenchymal-to-epithelial transition (MET) (182).

However, Imaoka *et al.* (183) also evaluated serum EMT-associated miRNAs as metastatic biomarkers by analysing the expression levels of the miR-200 family and miR-203 in serum from stage I ($n = 12$) and stage IV ($n = 12$) gastric cancer (GC) patients. They performed a validation phase in serum samples from 130 patients and 22 controls. They found that serum miR-203 expression was lower in GC patients with a higher T stage, vessel invasion, and lymph node and distant metastases; therefore, miR-203 has the potential to serve as a noninvasive biomarker to predict metastasis. However, no further studies were performed. Regarding the endogenous expression of this miRNA, recent studies reported that forced expression of miR-203 was associated with inhibited proliferation and reduced invasion and induction of MET in cancer cells from neck, laryngeal and tongue cancer. However, one study reported that miR-203 promoted proliferation and invasion in pancreatic cancer cells (184). Perhaps, studies regarding miR-203 expression in metastatic and matching primary tumour tissue, as well as *in vitro* studies in invasive and non-invasive clones of cancer cells, would provide more clues regarding its participation in metastasis.

Another recent study by Stückrath *et al.* (185) reported that plasma levels of miR-16, miR-107, miR-130a and miR-146a were decreased in lymph node-positive patients compared with lymph node-negative breast cancer patients; therefore, they could be potential biomarkers of metastasis. The authors further examined the effect of miR-107 expression on cell migration and invasion in breast cancer cell lines. They found that miR-107 overexpression reduced migration and

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invasiveness of both non-invasive MCF-7 and invasive MDA-MB-231 cells, while miR-107 inhibition increased migration of MCF-7 cells and invasiveness of both cell lines. This study did not further analyse the molecular mechanisms involved in the observed effect. Nevertheless, another recent study regarding endogenous miR-107 reported that its overexpression increased the migration of the hepatocarcinoma cell lines HepG2 and Huh7 through its target CPEB3 that acts *via* the EGFR pathway (186). This discrepancy could be an example of different miRNA functions that depend on the specific target gene and the specific type of tumour cell analysed. Moreover, observed miRNA function could depend on the type of miRNA expression: secreted or endogenous. Nevertheless, several circulating miRNAs have been recently identified as potential biomarkers for cancer metastasis (Table 4) (107,187–198).

The participation of cell-free miRNAs in metastasis has also been analysed in *in vitro* models. As was previously mentioned, miR-10b was highly secreted *via* exosomes by a metastatic breast cancer cell line compared with a non-metastatic breast cancer cell line and non-malignant breast cells (147). The authors showed that treatment with miR-10b-enriched exosomes suppressed protein levels of its target genes HOXD10 and KLF4 in the non-metastatic breast cancer line HMLE, inducing acquired invasion ability. However, they did not report the levels of circulating or endogenous miR-10b in clinical samples. Although, another study by Xu *et al.* (199) reported that serum mR-10b could be used as a biomarker to distinguish oesophageal cancer patients from healthy subjects. Another example is the study by Ostenfeld *et al.* (200). The aim of the study was to determine whether exosomal miRNAs were associated with the metastatic properties of bladder carcinoma cells.

Table 4. Cell-free microRNAs as biomarkers for cancer metastasis

Body fluid	Cancer	miRNAs	Biomarker for	References
Plasma	CRC	miR-96, miR-203, miR-141 and miR-200b	miR-96 distinguished stage I–IV CRC from HC; miR-203 distinguished stage III–IV CRC patients from stage I–II; miR-141 differentiated stage IV CRC from stage I–III patients; miR-96 and miR-200b were independent prognostic factors for overall survival	187
Plasma	BC	miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p	Onset of metastasis	188
Plasma	Breast cancer (BC)	miR-16, miR-107, miR-130a and miR-146	Predicts lymph node metastasis	185
Serum	GC	miR-203	Predicts metastases, early recurrence and poor prognosis	183
Serum	Small-cell lung cancer (SCLC)	miR-184, miR-574-5p, miR-874, miR-3074-5p, miR-4459, miR-4685-5p and miR-4746-3p	SCLC metastasis	189
Cerebrospinal fluid	Glioma	miR-21	Tumour spinal/ventricle metastasis	107
Plasma	Gastric cancer (GC)	miR-214	GC diagnosis and distant metastasis	198
Serum	Cholangiocarcinoma (CCA)	miR-106a	Higher risk of metastasis to lymph node	190
Plasma	Hepatocellular carcinoma (HCC)	miR-101	Distant metastatic	191
Serum	Melanoma	miR-210	Early systemic melanoma recurrence	192
Serum	Colorectal cancer (CRC)	miR-200c	Predicts metastatic phenotype, lymph node metastasis, liver metastasis and the development of distant metastasis	174
Serum	GC	miR-218	Metastasis	193
Plasma	Nasopharyngeal carcinoma (NPC)	miR-9	Metastasis	194
Serum	Cervical squamous cell carcinoma (CSCC)	miR-1246, miR-20a, miR-2392, miR-3147, miR-3162-5p and miR-4484	Predictive for lymph node metastasis in patients with early-stage CSCC	195
Serum	GC	miR-21, miR-146a and miR-148a	Metastasis to lymph node	196
Serum	Melanoma	miR-29c and miR-324-3p	Metastasis	197

Ostenfeld *et al.* (200) first found a relative increase in the secretion of exosomal miRNAs previously associated with tumour-suppressor functions (including miR-23b, miR-224 and miR-921) using SLT4 (metastatic) versus parenteral T24 (non-metastatic) cells and LUL2 (high metastatic) versus UMUC3 (low metastatic) cells. Then, they ectopically expressed miR-23b in T24 and FL3 cells and observed that invasion capacity was reduced for metastatic FL3 cells and increased for non-metastatic T24 cells. They further examined early lung metastasis in NCr nude mice upon tail injection of FL3-GFP-miR23b and FL3-GFP (control) cells, and they observed a reduced tumour cell burden for FL3-GFP-miR23b. The authors suggested that the tumour suppressor miR-23b was secreted by metastatic carcinoma cells for disposal and to improve the metastatic capacity of parental cells as a secondary effect. Notably, this study provided evidence that the same miRNA can act differently in a specific type of cell with different metastatic abilities.

Angiogenesis

Meanwhile, there are little more studies that analysed the participation of circulating miRNAs in angiogenesis. Angiogenesis is another vital process associated with tumour progression and metastasis. Angiogenesis is the physiological process of the formation of new capillaries from pre-existing vessels that is initiated by ischaemic and hypoxic conditions. Aberrant tumour angiogenesis occurs when rapidly proliferating cancer cells outgrow their blood supply and induce tumour hypoxia. Tumour hypoxia and excessive growth-promoting signals produced by tumour cells induce persistent and unresolved angiogenesis. Therefore, angiogenesis is vital for the survival, proliferation and propagation of tumour cells.

Zhuang *et al.* (201) reported that miR-9 was secreted by tumour cells and promoted endothelial cell migration and angiogenesis. They used co-cultures of tumour cell lines and matching endothelial cells from normal tissue to discover that miRNAs packaged into microvesicles, and not known growth factors, promoted endothelial migration. Then, they found that miR-9 was a relevant miRNA by inhibiting its expression in tumour cells and observed that the effect was retained using the conditional media from tumour cells. Overexpression of miR-9 in endothelial cells reduced SOCS5 levels, leading to activation of the JAK-STAT pathway, which promotes cell migration. In addition, they used a mouse model of subcutaneous implantation of the human colorectal carcinoma cell line HM7, which resulted in elevated levels of miR-9 in the plasma, and found that intratumoural injection of miR-9 antagomirs significantly decreased tumour angiogenesis.

However, Yamada *et al.* (202) reported that colorectal cancer cells secreted microvesicles containing miR-1246 and TGF-β that promoted angiogenesis by activating Smad 1/5/8 in human umbilical vein endothelial cells, inducing proliferation, migration and tube formation. Kosaka *et al.* (203) showed that exosomal miR-210, which was released from metastatic breast cancer cells *via* an nSMase2-dependent secretion mechanism, enhanced angiogenic activity in endothelial cells. These authors found that the known angiogenic miR-210 was highly enriched in exosomes from the metastatic cell line (MDA-MB-231) compared with the non-metastatic cell line (MCF7) and normal mammary epithelial cells (MCF10A). To show that exosomal miR-210 contributed to the enhancement of endothelial angiogenic activity, they used miR-210-enriched exosomes from miR-210 transiently transfected 4T1 cells to induce capillary formation and migration of HUVECs. Accordingly, the levels of plasma miR-210 were found to be increased in breast cancer patients with lymph node metastasis in another study (204).

Meanwhile, Li *et al.* (205) implanted mouse sarcoma S-180 cells into C57BL/6J mice and found that the plasma levels of exosomal miR-150 of the tumour-implanted mice were higher than those of the control mice. Using Matrigel plugs, they found that tumour implantation stimulated angiogenesis and that this angiogenic effect was suppressed in mice treated with microvesicles containing a miR-150 inhibitor. They initially reported that microvesicles containing high levels of miR-150 were secreted by the human monocyte cell line THP-1 and enhanced the tube formation of endothelial HMEC-1 cells.

There are other studies that reported that exosomes affected tumour angiogenesis and metastasis (206,207), but they did not analyse the participation of the miRNAs contained in such exosomes.

Evasion of immune response

The immune system is able to distinguish cancer cells from normal cells and eliminate them. Nevertheless, when cancer cells evade the anti-tumour immune surveillance, they grow progressively and take advantage of the proliferative and angiogenic signals delivered during the immune response (208). Recent studies have reported that dysregulation of certain endogenous miRNAs in the tumour microenvironment may contribute to immune evasion (209–212); however, only one study regarding secreted miRNAs has been published (120).

Ye *et al.* (120) examined the role of tumour-derived exosomes in tumour progression in nasopharyngeal carcinoma (NPC). This study reported that tumour-derived

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exosomes promote T-cell dysfunction through the regulation of enriched exosomal miRNAs. However, the data showed that NPC-derived exosomes impeded the proliferation of T cells and the differentiation of Th1 and Th17 and altered the cytokine profiles of stimulated lymphocytes without assessing that the enriched miRNAs found in exosomes participated in these effects. After obtaining experimental evidence of the effect of tumour-derived exosomes on T cells, the authors found that five miRNAs, including miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p and miR-1908, were commonly enriched in the exosomes from the serum of NPC patients and in TW03 or TW03 NPC cells. Then, they performed a bioinformatics analysis that predicted that the mitogen-activated protein kinase (MAPK) signalling pathway was associated with those miRNAs.

There are other publications regarding the role of tumour-derived exosomes in immune evasion, but they did not analyse whether the miRNAs contained in those exosomes were responsible for the observed effect on the immune response (213,214).

Regulation of miRNA expression

Alterations in the expression levels of miRNAs have been associated with the pathogenesis of several human diseases, including cancer. Therefore, understanding the regulation of the expression of miRNAs is relevant to elucidate which mechanisms are involved in dysregulation of miRNA expression. However, studies have only recently provided clues about this crucial topic. This section briefly describes some mechanisms of regulation of miRNA expression and the causes of their dysregulation in cancer. There are extensive reviews about this topic elsewhere (215,216).

miRNA transcription and dysregulation by transcription factors

Most miRNAs are transcribed by RNA polymerase II (217); therefore, they are subjected to the same regulatory mechanisms as mRNA transcripts. miRNA promoters contain CpG islands, TATA boxes and TFIIB recognition sites, and histone modifications have been observed within them (217). Additionally, the expression of primary miRNA transcripts was found to be regulated by several transcription factors. For example, c-Myc is an oncogene that is frequently dysregulated in cancer and was found to bind to E box elements in the *miR-17~92* gene promoter, inducing the transcriptional activation of miR-17~92 (218). The cluster miR-17~92 promotes cell-cycle progression and proliferation, through the regulation of E2F transcription factors that regulate cell-cycle progression (219), and the suppression of the tumour suppressor Pten and the pro-apoptotic protein Bim (220). The miR-17~92 is clustered within the region of C13orf25 transcript variant 2, which is a target for 13q31-q32 amplification in malignant lymphoma (221). It was found that genomic amplification of this region correlated with overexpression of five members of the miR-17~92 cluster in malignant lymphoma cell lines (222). Additionally, c-Myc negatively regulates the transcriptional activity of tumour suppressor miRNAs, such as miR-15a, miR-34 and the let-7 family of miRNAs (223). Another example is p53, which binds to the promoter of miR-34a and miR-34b/c and induces their expression (224). The miR-34 family of miRNAs, including miR-34a/b/c, promotes cell-cycle arrest, cell senescence and apoptosis in cancer (225). Other regulatory pathways that affect the expression of miR-34a in cancer include CEBPa, which when mutated induces decreased miR-34a levels and enhanced expression of E2F3, resulting in acute myeloid leukaemia (226).

Methylation of miRNA promoters

MicroRNA genes can be epigenetically silenced through DNA methylation. Saito *et al.* (227) reported for the first time that miRNAs were strongly upregulated after treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine in T24 bladder cancer cells. Among them, miR-127 was later found to be downregulated in primary prostate, bladder and colon cancer tissues compared with matched normal tissues. Further evidence indicated that miR-127 functions as a tumour suppressor by targeting the proto-oncogene BCL6.

Another example of epigenetic silencing of tumour suppressor miRNA is the study by Toyota *et al.* (228). They found that miR-34b and miR-34c were epigenetically silenced in colorectal cancer by hypermethylation of the neighbouring CpG island. Methylation of the miR-34b/c CpG Island was observed in colorectal cancer cell lines and primary tumours but not in normal colonic mucosa. Overexpression of miR-34b/c suppressed colony formation of HCT116 cells.

MiRNA editing

RNA editing is the site-specific modification of an RNA sequence to yield a product differing from that encoded by the DNA template (229). Most RNA editing in human cells is adenosine-to-inosine (A-to-I) RNA editing and is catalysed by the adenosine deaminases acting on RNA (ADARs). Blow *et al.* (229) identified novel A-to-I editing sites in 6 of 99 pri-miRNAs, using an assay that detect relatively high levels of editing.

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Previously, Luciano *et al.* (230) had shown that the pri-miRNA transcript of miR-22 is subject to A-to-I RNA editing in a number of human and mouse tissue (230). Editing of pri-miRNAs would have major effect for miRNA biogenesis and function, because DICER activity depends on proper base pairing within the stem region, and editing within the 20–22mer portion of the mature miRNA would alter the target mRNA repertoire for that particular miRNA. It was hypothesized that disruption to the A-to-I editing of the transcripts would lead to altered gene expression profiles in cancer that regulate tumour phenotypes and clinical behaviours. The study by Choudhury *et al.* (231) demonstrated that the A-to-I editing of the miR-376 cluster miRNAs is significantly reduced in high-grade human gliomas, resulting in accumulation of the unedited form in glioblastoma cells. Furthermore, authors showed that the overexpression of unedited miR-376a* in glioblastoma cells promoted their migration and invasion while edited miR-376a* suppressed this ability *in vitro*. It was shown that unedited miR-376a* targeted RAP2A (a member of the RAS oncogene family), which produces a protein involved in both cancer cell migration and axomal branching, while the edited miR-376a* targeted autocrine motility factor receptor (AMFR). For more information regarding miRNA processing, refer Schmittgen (232).

Conclusions

The potential usefulness of cell-free miRNAs as reliable biomarkers in cancer may be based on their biological characteristics and potential functions. Evidence indicates that miRNAs are released from normal and tumour cells into the blood and other fluid in highly specific patterns and through mechanisms that depend on the physiology of each cell type. Such specificity suggests that miRNAs in fluids might not be the result of passive leakage from broken cells, but rather might be due to specific cellular transport mechanisms.

Currently, miRNAs are thought to be released to the outside environment for cell-to-cell communication. Like their intracellular counterparts, cell-free miRNAs function as key regulators of gene expression, and disrupting or altering them could lead to carcinogenesis. The discovery that extracellular miRNAs are involved in carcinogenesis has led to potential applications to determine cancer diagnosis and prognosis and could even play a role in cancer therapy. Easy access to cell-free miRNAs and their relative stability are important advantages for their use as biomarkers; however, reliable miRNA biomarkers still need to be identified, considering that their levels appear to depend on several normal and pathological physiological conditions. For their use as biomarkers, it is essential to determine the biological characteristics, release mechanisms/external forms and functions in oncogenesis of cell-free miRNAs.

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