

Review

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Circulating cell-free microRNAs as clinical cancer biomarkers

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Abstract: MicroRNAs (miRNAs) are non-coding small RNAs that are master regulators of genic expression and consequently of many cellular processes. But their expression is often deregulated in human tumors leading to cancer development. Recently miRNAs were discovered in body fluids (serum, plasma and others) and their levels have often been reported to be altered in patients. Circulating miRNAs became one of the most promising biomarkers in oncology for early diagnosis, prognosis and therapeutic response prediction. Here we describe the origins and roles of miRNAs, and summarize the most recent studies focusing on their usefulness as cancer biomarkers in lung, breast, colon, prostate, ovary cancers and melanoma. Lastly, we describe the main methodologies related to miRNA detection, which should be standardized for their use in clinical practice.

Keywords: biomarkers; cancer; microRNA.

Introduction

Identified 20 years ago, microRNAs (miRNAs) are non-coding single strand small RNA molecules of about 22 nucleotides in length that act as post-transcriptional regulators of gene expression and control many critical cellular processes. Numerous studies have reported aberrant expression of miRNAs in a range of different pathologies, with striking alterations in tumor tissues (1). Profiling of miRNAs has contributed to the molecular classification

of tumors according to cancer type and prognosis (2). In 2008, the presence of miRNAs was reported in body fluids (urine, serum, plasma, etc. ...) allowing non-invasive identification of individuals with cancer (3–5). Exponentially growing evidence shows that measurements of miRNAs in serum or plasma can provide valuable non-invasive biomarkers for detection of various human cancers (6, 7). Herein, a general overview of the utility of circulating miRNAs as cancer biomarkers will be presented with an emphasis on the more recent findings on several types of cancer.

Biogenesis of miRNAs

miRNAs are transcribed in the nucleus by RNA polymerase II as long primary microRNA (pri-miRNA) precursor molecules whose lengths vary greatly (up to 3–4 kb miRNAs) (8). They are then processed by the ribonuclease III Droscha associated with a partner called DGCR8, into pre-miRNAs, 60–70 nt long (9), that are exported to the cytoplasm by exportin 5 and its partner Ran-GTP (10). The second RNase III Dicer removing the loop of the pre-miRNA then generates a small double strand RNA of about 22 nt. Dicer is associated with a catalytic complex called the RNA-induced silencing complex (RISC) for miRNA-mediated post-transcriptional gene silencing with the transactivation responsive RNA-binding protein (TRBP) that enhances the fidelity of the cleavage and recruits to the Argonaute (AGO) proteins, the catalytic engine of RISC (11). AGO loads the mature strand of the miRNA, while the passenger strand is dissociated and degraded (12), resulting in a fully active miRNA [for an extensive review, see Ref. (13)]. In the past, both the strands of a microRNA gene were named miR and miR*, the asterisk indicating that the miR is considered as a ‘minor’ product, found at lower concentration, and inferred that miR* is non-functional. But several miR*s have proven to be functional. For clarification a new nomenclature was adopted. miRNAs originating from the 3’ end or 5’ end of the microRNA gene are denoted with a ‘-3p’ or ‘-5p’ suffix, respectively.

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microRNA functions

The binding of the complex RISC to mRNA is mediated by a sequence of 2–8 nucleotides, known as the seed region, at the 5' end of the mature miRNA (14). The complex binds to the target mRNA, via its partially complementary sequence, most often in the 3' but sometimes in the 5' untranslated region (15), in the open reading frame (16) or in the promoter regions (17). miRNAs inhibit gene expression through several mechanisms, depending on the degree of complementarity between the small RNA and its mRNA target (18). It was reported that the mRNA cleavage is induced when the homology is perfect (19), but mRNAs containing partial miRNA complementary sites can also be targeted for degradation *in vivo* (20). miRNAs could also induce repression of mRNA translation, at the level of translation initiation (21) and as of post-initiation (22). It has also been shown that miRNAs can upregulate the expression of their target genes (23). For extensive information on the modes of miRNA actions, see the reviews of Morozova et al. (24) and James et al. (25).

A single miRNA can target up to a hundred genes due to the imperfect matching outside the seed sequence (26) and conversely a single mRNA could be controlled by several miRNAs. The latest version of miRBase (release 21) has annotated 2588 human mature miRNAs sequences, which can target more than 30% of the genome (27). An analysis has even suggested that more than 80% of the gene transcripts are likely under microRNA control through their untranslated and amino acid-coding regions (28). Therefore the miRNAs play a critical role in multiple biological processes including proliferation, differentiation, apoptosis and hematopoiesis (29). Thus it is quite obvious that a dysregulation of miRNA expression leads to a number of pathologies such as inflammation, cardiovascular diseases, neurological disorders and several types of cancer.

miRNAs in cancer

miRNAs contribute to cancer by regulating either oncogenes (tumor suppressor miRNA) or tumor suppressors (oncomiRs). Most of the time oncomiRs, such as miR-17-92 cluster (30) or miR-21 (31), are overexpressed and tumor suppressor miRNAs, such as let-7 family, are downregulated (32). Gain and loss of function experiments have provided insights into the role of miRNA in oncogenesis. For instance, enforced expression of the miR-17-92 cluster that codes for miR-17-3p, -17-5p, -18a, -20a, -19a, -19b and -92,

participates in the tumor development in a mouse B-cell lymphoma model by inhibition of the apoptotic pathway and cell cycle (30). In contrast, early studies showed that overexpression of miRNAs of the let-7 family-inhibited tumor formation, progression and metastasis can induce apoptosis through targeting many signaling pathways (RAS, c-MYC, cyclin D1/2/3, cyclin A, CDK4/6, etc. ...) (33). But more recent studies show oncogenic functions of let-7 repressing the tumor-suppressive caspase-3 and BAX genes (34, 35). Several other miRNAs can act as oncomiR as well as tumor suppressor depending on the context (36). For instance, miR-155 was often considered as an oncomiR in different cancer types (36) such as pancreatic cancer and lymphoma (37, 38) and its overexpression induces B cell malignancy in mice (39). However, several groups reported that miR-155 displays tumor suppressive role in melanoma, gastric and ovarian cancers where it is downregulated (40–42).

Dysregulated miRNAs expression causes a loss of control of critical biological processes – proliferation, differentiation, apoptosis, EMT, migration – leading to oncogenesis. miR-21, a miRNA ubiquitously upregulated in cancer is the best example of this (43). miR-21 affects all major pathways of carcinogenesis (proliferation, apoptosis, angiogenesis and invasion), through its multiple targets including *PTEN* (phosphatase and tensin homolog) (44), *PDCD4* (tumor suppressor gene tropomyosin 4) (45) *FasL* (pro-apoptotic FAS ligand) (46), and *TIMP3* (metalloproteinase inhibitor 3 precursor) (47). Many miRNAs are now known to be regulators of metastasis, interfering with the different steps of the metastatic cascade (cell adhesion, migration, EMT, etc.) (48). The miR-200 family is well known as a regulator of EMT, targeting key transcription factors such as ZEB-1 and ZEB-2 (49). The transcription of miR-10b is positively regulated by Twist 1 and in turn increases the expression of RHOC, involved in metastasis (50). Similarly overexpression of miR-21 promotes a metastatic phenotype by targeting the tumor suppressor RHOB (51).

The biogenesis of miRNAs is a tightly controlled process but it has become evident that miRNAs are deregulated in cancers. Calin et al. were the first to report a deregulation of miRNAs in cancer (52). They showed that the loci miR-15-16 is deleted in patients with B cell chronic lymphocytic leukemia. Then an exponentially growing number of publications showed that miRNAs expression is altered in cancer (53–56). The causes of miRNA aberrant expression in cancer are multiple. Half of the miRNA genes are localized in fragile sites or in cancer-associated genomic regions amplified or translocated in cancer such as miR-15 and miR-16 on the 13q14 locus (57). A CGH array screening

227 tumors has shown that 37.1%–85.9% of miRNAs exhibit DNA copy number alterations, correlating with miRNA expression (58). Moreover an alteration of the expression or function of enzymes of the miRNA processing, such as Droscha, Dicer 1 or DGCR8, was reported (59). A decrease of Dicer or Droscha has been observed in ovarian cancer associated with poor survival (60) and in bladder cancer (61) while their upregulation occurs in cervical squamous cell carcinoma (62), and gastric cancers (63). An analysis of gene expression in primary tumors indicates that the widespread downregulation of miRNAs observed in cancer is due to a failure at the Droscha-processing step (64). Somatic mutations of the microRNA-processing enzymes such Droscha, DGCR8 or Dicer 1 has also been observed notably in neuroblastomas (65). Splice variants of Droscha encoding truncated proteins were found in melanoma (66). Many studies indicate that the microRNA expression may be also regulated by different epigenetic mechanisms leading to the silencing of the tumor suppressor microRNA including abnormal methylation of the promoter regions (67) or histone modifications (68). Another important level of regulation of microRNA expression is its transcriptional control. The alteration of activators or repressors of pri-miRNA transcription results in miRNA level defects. The miR-34 family is known to be regulated by p53 and to be partially responsible for the onco-suppressor-induced phenotype (59, 69). Myc regulates the transcription of the oncogenic miR-17-92 cluster (70, 71) while Twist1 suppresses let-7i via binding to its promoter, to activate mesenchymal mode migration (50, 72) and c-Met via the transcription factors c-Jun and AP1 induces the oncomiR miR-221-222 cluster (73). Forkhead box (FOX) transcriptions factors as well as Hippo-signaling pathway were recently shown to regulate the miRNA transcription as well (74, 75).

Circulating miRNAs as biomarkers in cancer

Several profiling studies with microarrays, among them the study of Rosenfeld and coworkers who investigated 400 samples from 22 different tumors and metastases, have shown that miRNAs' expression signatures permit, with high accuracy, tumor classification, according to the tissue of origin (76–78). The tumor tissues could be distinguished from normal tissues in CLL (79) lung (80, 81), breast (82), or prostate cancer (PCa) (83, 84). Moreover, besides its diagnostic utility, it turns out that the profiling of miRNAs might also be a useful tool for prognosis, prediction of metastatic outcome and therapeutic response (78, 85–87).

In 2008, princeps studies reported the presence of miRNAs in plasma and serum (3–5, 88) and that, between healthy donors and those patients with cancer or diabetes, the profiles of serum miRNAs differ (3). It was then observed that miRNAs were present in all of the 12 body fluids assessed, including plasma, urine, saliva, peritoneal fluid, pleural fluid, seminal fluid, tears, amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid and colostrum (89). The concentration and the profiles of the miRNA vary between the diverse fluids. Human urine has the lowest concentration and diversity of miRNA while breast milk displays a huge concentration and a number of miRNAs (89). On the other hand, some reports described higher miRNA concentrations in serum samples compared to the corresponding plasma samples (5, 90) in contrast to results shown by McDonald et al. (91). The discrepancy between these observations may be the result of an miRNA release from blood cells during the coagulation process (90).

In the blood, the circulating miRNAs are packaged in extracellular vesicles – microvesicles, exosomes or apoptotic bodies – (92) or complexed to RNA-binding proteins, AGO2 (93, 94) or nucleophosmin (95), but also to HDL (96, 97). It has been proposed that a large majority of plasma miRNAs are complexed with AGO proteins, while the miRNAs packaged into vesicles are poorly represented (93–95), but other studies contradicted these results (97–99). The precise mechanisms of the release of the miRNAs into extracellular compartment are not yet completely understood. The miRNAs could be released by a passive mode in pathological conditions such as necrosis, apoptosis or inflammation or by an active and selective process or a combination of both (100). The exosomal miRNAs appear to be selectively recruited and actively secreted in a regulatory manner (48, 101). Indeed the exosomal and donor cell miRNA profiles differ as reported in several publications (102–105). Some miRNAs are concentrated in exosomes while the expression of most of them is lower in exosomes vs. cells (102, 106). While the precise mechanism of the regulation of the miRNAs release is not yet fully deciphered, RAB proteins, essential regulators of intracellular vesicle transport, have emerged as the key regulators of exosome secretion (107). Moreover it has been proposed that the exosome secretion is triggered by a ceramide-dependent pathway (6, 105, 108).

The circulating extracellular miRNAs are believed to play an important role in intercellular and inter-organ communication. Several mechanisms of internalization of extracellular miRNAs by recipient cells have been proposed (109, 110). They could be internalized to elicit their regulatory functions, notably either (i) by endocytosis, phagocytosis, or by direct fusion of the vesicles with the

plasma membranes of the recipient cells, or (ii) by uptake by cell surface receptors of the complexes with AGO2. Extracellular miRNAs are able to promote multiple biological processes in the recipient cells and tissues, such as proliferation, invasion, metastasis and angiogenesis (48, 110–112). But a recent study has challenged the existence of exosomal miRNAs (113). By using a new exosome quantification technique, Chevillet et al. (113, 114) have observed that most of them do not carry any miRNAs, bringing into question their involvement in cell-cell communication.

However, it is now well established that the circulating miRNAs are linked to cancer and appear to be promising diagnostic and prognostic, non-invasive biomarkers in various cancers. Indeed, the circulating miRNAs display several meaningful properties making them good potential biomarkers. They show a remarkable stability in bodily fluids, where they are protected from endogenous RNase's activity by vesicles or carrier proteins, while miRNAs added exogenously are quickly degraded (3–5). The circulating miRNAs resist prolonged incubation at room temperature and to multiple freeze-thaw cycles (5). Moreover, the circulating miRNAs show constant homogeneous expression in healthy individuals, derived essentially from blood cells (3). By contrast, in cancer patients, most of the circulating miRNAs appeared to be directly derived from tumor tissues and may reflect the tumor burden. Indeed, in cancer patients, the plasma or serum miRNA profiles correlate with the tumor tissues' profile. On the other hand, several studies demonstrated that circulating oncogenic miRNA levels decreased after tumor resection (115).

A recent survey of 148 published reports in the eight most prominent cancers reports a total of 279 deregulated circulating miRNAs in serum and plasma from cancer patients to healthy donors (116). A tremendous number of publications proposed that the circulating miRNAs, especially in serum and plasma, could potentially be used as diagnostic, prognostic and predictive biomarkers for different types of tumors (6, 29, 100, 116–123).

It is well established that the early detection of cancer significantly improves outcomes for patients. Late diagnosis is one of the most prevalent reasons for the high mortality rate in cancer notably in lung cancer. Thus, very sensitive and specific tools are needed. Currently the classical diagnostic methods, such as CT-scan and mammography are expensive, could be dangerous when repeated, and their specificity and sensitivity are not optimal. Moreover, in the era of personalized therapies, the need for non-invasive biomarkers to get iterative information on the pathology is critical and has led to the research of circulating biomarkers since repeated biopsies are not feasible and often associated with morbidity. A rapid and

non-invasive access to the molecular profile of tumors is a current challenge. The liquid biopsies, circulating tumor cells, circulating-free DNA, and miRNAs isolated from the blood of patients emerged as potential tools and are one of the most active areas of translational research in several types of cancer.

Lung cancer

Lung cancer is the leading cause of cancer deaths in developed countries, with non-small cell lung cancer (NSCLC) that accounts for the majority of cases. Late diagnosis is one of the most prevalent reasons for the high mortality rate. The overall 5-year survival rate is no more than 15%. Besides the need for early detection of pathology, a non-invasive way to characterize the molecular profile of tumors over time is required given the increasing number of targeted therapies available for the treatment of NSCLC and its dynamic changes through cancer treatment.

Several original publications and reviews have reported that the levels of multiple miRNAs are altered in lung cancer. Recently Zhao et al. (124) have listed among different studies from 2011 to 2015, 39 miRNAs upregulated and 18 miRNAs downregulated, that correlated notably with clinical stages, metastasis or early lung cancer. A meta-analysis based on 28 publications with a total of 2121 patients and 1582 healthy ones shows that miRNA may serve as a potential biomarker in NSCLC detection, especially from blood, with a high diagnostic accuracy (125). Moreover, Ulivi and colleagues have analyzed 28 publications between 2009 and 2014 that compared microRNA levels in serum, plasma and sputum from lung cancer patients to healthy donors and summarizes the promising miRNAs for lung cancer diagnosis (126).

Many other excellent reviews have described the role of miRNAs notably in diagnosis, prognosis and therapeutic response in lung cancer (29, 100, 116, 117, 119, 122, 124, 126–130). Herein we have tried to summarize the most recent publications in Table 1. It is noteworthy that in a large proportion of the publications, miRNA panels are used rather than a single miRNA to discriminate with higher specificity and sensitivity, patients with lung cancer and healthy controls.

For example, in 2011 Bianchi and coworkers developed a test, based on the detection of 34 miRNAs from serum, that could identify patients with early stage NSCLCs in a population of asymptomatic high-risk individuals with 80% accuracy (131). Later, the authors refined this signature to 13 miRNAs maintaining the same performance in order to reduce the costs and complexity of the test

Table 1: Lung cancer circulating miRNAs.

miRNA	Expression	Sample source	Patient cohort ^a	Healthy controls ^b	Type assay	Reference miRNA	Role	AUC ^c	Reference
- Panel with miR-125a-5p, miR-25 and miR-126	Down	Serum	94 I-II, 48 III-IV	111 HD	Meta-analysis	Cel-miR-39	Early detection	0.936	(133)
- miR-31	Up	Plasma	300 I to IV	300 HD	RTqPCR	miR-16	Diagnosis and prognosis	0.785	(134)
- Panel with 24 miRNAs		Plasma	69	870 HD	RTqPCR	miRNA ratio	Diagnosis, prognosis and prediction		(135)
- miR-Test : Panel with 13 miRNAs including miR-92a-3p, miR-30b-5p, miR-191-5p, miR-484, miR-328-3p, miR-30c-5p, miR-374a-5p, let-7d-5p, miR-331-3p, miR-29a-3p, miR-148a-3p, miR-223-3p, miR-140-5p		Serum	1115 high risk patients		RTqPCR	miR-197, miR-19b, miR-24, miR-146, miR-15b, miR19a	Early detection	0.85	(132)
- Panel with 24 miRNAs		Plasma	100 I-IIIa	100 HD	Taqman microarray RTqPCR	Spiked Arabidopsis thaliana miR-159a RNU6	Diagnosis	0.94	(136)
- Panel with miR-448 and miR-4478	Up	Plasma	65 NSCLCC (40 I-IIIa, 25 IIIB-IV) 25 SCLC	85 HD	RTqPCR		Early diagnosis	0.896	(137)
- Panel with miR-148a, miR-148b, miR-152 and miR-21		Serum	34 I-II, 118 III-IV	300 HD	RTqPCR	RNU6	Diagnosis	0.97	(138)
- miR-125a-5p	Up	Serum	70	70 HD	RTqPCR	Cel-miR-39	Diagnosis	0.7	(139)
- miR-148	Up							0.84	
- miR146a	Up							0.78	
- Let-7c	Down	Plasma	69 I, 51 II to IV	360 HD	RTqPCR	RNU6	Diagnosis	0.714	(140)
- miR-152	Down							0.845	
- Panel with miR-20a, miR-16 and miR-15		Serum	116 I, 48 IIA-IIIB	124 HD	RTqPCR with calibration curve	-	Diagnosis	0.93	(141)
- Panel with miR-23b, miR-10b-5p and miR-215p		Plasma exosomes	100 I-IIIa, 87 IIIB-IV, 9 unknown		qPCR microarray RTqPCR	Let-7a-5p	Prognosis	0.91	(142)
- miR-1246	Up	Serum	59 I-III	65 HD	Microarray RTqPCR	miR-425-5p, miR-16 and RNU48	Diagnosis and surrogate marker of therapy response	-	(143)
- miR-1290	Up								
- miR-195	Down	Plasma	100 I-III	100	RTqPCR	Cel-miR-39 RNU6	Prognosis	0.89	(144)
- Panel with miR-141, miR-193b, miR-200b and miR-301	Up	Serum	70 I-III, 84 IV	22 HD	Tasman open array RTqPCR		Early detection	0.985	(145)
- Panel with miR-25, miR-122, miR-195, miR-21 and miR-125b		Plasma	90 wild-type EGFR, 59 Mutated EGFR	23 HD	Microarray RTqPCR	RNU6	Discrimination EGFR mutation	0.993	(146)

^aNumber in validation sets; ^bAUC for area under the receiver-operating characteristic curve.

and to increase its clinical translatability. This test called miR-Test was validated on a large-scale validation cohort of high-risk patients (n=1115) and showed a sensitivity and specificity of 77.8% and 74.8%, respectively (132). As shown in Table 1, many other signatures appear to be useful and promising tools for diagnosis, prognosis or as a surrogate marker for therapy response. But few overlaps could be found between the different studies.

Breast cancer

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012. This represents about 12% of all new cancer cases and 25% of all cancers in women (147). Mammography and ultrasound imaging are widely used for detecting breast cancer and have helped to improve overall survival, but they are known to have a limited specificity and sensitivity. Moreover breast cancer is a very complex and heterogeneous pathology, determined by established markers such as size, node and IHC profiling of hormone receptors (ER, PR, HER2) status and proliferation marker Ki-67. These markers along with two serum-based tumor biomarkers (CA15-3 and CEA) are used to evaluate individual prognosis but with limited sensitivity and specificity. Reliable blood-based biomarkers are needed to assess prognosis but also diagnosis and response to therapy.

Numerous studies have focused on circulating miRNAs as biomarkers in breast cancer diagnosis, classification and prognosis and reviewed by several teams (29, 100, 116, 117, 119, 122, 148–153). He et al. have analyzed 35 publications with 2850 breast cancer patients and 1479 health controls, and identified 106 (61 in plasma and 45 in serum) deregulated circulating miRNAs but only 15 among them miR-21 have been reported by more than one study (116). Recently Li et al. performed a meta-analysis of six studies with 438 patients and 228 healthy controls and indeed showed that miR-21 could be an accurate biomarker for early diagnosis (154). miRNA-155 was also reported as a diagnostic miRNA in breast cancer (153). Both these miRNA could be used also as a prognostic biomarker like miR-205 and miR-30a (153). miR-10b was associated with metastatic dissemination (155) and miR-210 or miR-155 to therapy response (151). As for lung cancer, many signatures of several miRNAs have also been identified as tools for early diagnosis, tumor staging or monitoring recurrence (Table 2). A recent study performed on 1206 cancer samples compared to healthy samples has found out a signature of five miRNAs (miR-1246, miR-1307,

miR-4634, miR-6861 and miR-6875) as an accurate tool for early detection of breast cancer (156).

Colorectal cancer

CRC is the third most common cancer and a leading cause of cancer-related death worldwide. Early diagnosis of CRC is a pre-requisite for proper management of the patient and increasing survival. Currently, colonoscopy is the gold standard for early diagnosis of CRC but its invasiveness is a big limitation; up to 12% of precancerous lesions miss detection and approximately 10% of CRCs occur in individuals within 3 years of a screening colonoscopy. Serum markers CEA and CA19-9 are used but are not sufficiently sensitive nor specific. Therefore, non-invasive and highly sensitive approaches are urgently needed for CRC screening. Several studies have shown that serum and plasma could detect CRC with high accuracy (100, 117, 119, 164, 165). He and coworkers have analyzed 25 studies with 2146 patients and 1267 healthy controls and found 78 deregulated miRNAs, among them miR-21, miR-29a and miR-15b were found in more than one study (116). A recent meta-analysis of nine studies has suggested miR-21 as a potential biomarker for CRC, with a pooled sensitivity and specificity of 72% and 85%, respectively (166) as shown previously (167). In contrast, Montagnana et al. have contested the use of miR-21 plasma levels as a diagnosis and staging CRC tool (168). Interestingly, it was reported that in addition to the changes in the level of the circulating miRNAs, miRNA polymorphisms could predict risk from CRC such as miR-146a polymorphism (Rs2910164) (169). Subgroup and meta-regression analyses of 19 articles demonstrated that multiple miRNA measurements display a higher predictive accuracy than a single miRNA in detecting CRC (170). In Table 3 we have summarized the most recent publications regarding the role of circulating miRNAs as diagnostic and prognostic tool in CRC. A panel of miRNAs (miR-31, miR-29c, miR-122, miR-192, miR-346, miR-372, miR-374c) was shown to have a very high specificity and sensitivity to discriminate CRC from adenoma when analyzed both in plasma and stool (171).

Melanoma

Melanoma is the deadliest form of skin cancer with an increasing incidence worldwide. Its early diagnosis is important because the majority of the localized stages are curable and cure rates are <15% for patients at AJCC stage IV (181). At present, there is no curative therapy for

Table 2: Breast cancer circulating miRNAs.

miRNA	Expression	Sample source	Patient cohort ^a	Healthy controls ^a	Type assay	Reference miRNA	Role	AUC ^b	Reference
– Panel with miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365 and miR-425)		Serum	36	80 HD	RTqPCR	miR-10b-5p	Early detection	0.61	(157)
– Panel of miR-16, miR-103, let-7d, miR-107, miR-148a, let-7i, miR-19b, miR-22*			149 primary cancer, 31 metastatic	133 HD	RTqPCR	Mean Cq of the 50 most expressed		0.81	(158)
– miR-10b	Up	Serum	50 0-I, 70 II-IV	50 HD	RTqPCR	miR-16	Tumors staging and detection of metastasis		(159)
miR-34a	Down								
miR-155a	Up								
miR-195	Up								
– miR-195	Down	PBMC	45 I-IV	60 HD	Taqman array	RNU6	Early detection	0.901	(160)
miR-495	Down				RTqPCR	miR-16		0.901	
– Panel with miR-199a, miR-29c, miR-424	Up	Serum	101 0-IIIa	72 HD	Multiplex RTqPCR	miR-103 and miR-132	Diagnosis	0.905	(161)
– Panel with miR-18b, miR-103, miR-107 and miR-652		Serum	110 triple negative	30 HD	RTqPCR	RNU6	Prognosis	0.81	(162)
– Panel with miR-1246, miR-1307-3p, miR-4634, miR-6861-5p and miR-6875-5p		Serum	1206 0-IV	1343 HD	Microarray RTqPCR	miR-149-3p, miR-2861 and miR-4463	Early detection	0.971	(156)
– Panel with miR-21-5p, miR-375, miR-205-5p, miR-194-5p, miR-382-5p, miR-376-3p and miR-411-5p		Serum	28 recurrent 62 non recurrent		RTqPCR	miR-361-5p and miR-186-5p	Monitoring recurrence	0.914	(163)

^aNumber in validation sets; ^bAUC for area under the receiver-operating characteristic curve.

Table 3: Colorectal cancer circulating miRNAs.

miRNA	Expression	Sample source	Patient cohort ^a	Healthy controls ^a	Type assay	Reference miRNA	Role	AUC ^b	Reference
- Panel with miR-23a-3p, miR-27a-3p, miR-142-5p, miR-376c-3p	Up	Serum	30 I, 67 II, 55 III, 51 IV	100 HD	RNA seq RTqPCR	miR-93-5p	Diagnosis healthy donors vs. all stages Diagnosis healthy donors vs. stages I/II	0.922 0.877	(172)
- miR-135a-5p	Up	Serum	60 cancer, 40 benign	50 HD	RTqPCR	RNU6	Diagnosis	0.875	(173)
- miR-210		Serum	38 I, 93 II, 126 III, 11 IV	102 HD	RTqPCR	miR-191-5p and RNU6	Diagnosis Prognosis	0.821	(174)
- Panel with miR-24, miR-320a and miR-423-5p	Down	Plasma	54 I-II, 57 III-IV	130 HD	RTqPCR	Cel-miR-39	Diagnosis	0.899	(175)
- Panel with miR-145, miR-17-3p and miR-106a		Serum	90 II-III	70 HD	TaqMan array RTqPCR	Let-7d/g/i	Early diagnosis Diagnosis Prognosis and therapeutic response prediction	0.941 0.886	(115)
- Panel with miR-17-3p and miR-106a		Serum	90 II-III	70 HD	TaqMan array RTqPCR	Let-7d/g/i	Early diagnosis Diagnosis Prognosis and therapeutic response prediction	0.941 0.886	(115)
- miR-106a		Serum	90 II-III	70 HD	TaqMan array RTqPCR	Let-7d/g/i	Early diagnosis Diagnosis Prognosis and therapeutic response prediction	0.941 0.886	(115)
- miR-223 and miR-92a		Plasma and stool	215	183 HD	Multiplex RTqPCR	Cel-miR-238	Diagnosis	Plasma only 0.78 Combination with stool 0.907	(176)
- Panel with miR-21, miR-29a and miR-125b		Serum	136 I-II	52 HD	RTqPCR	Cel-miR-39	Diagnosis	0.826	(177)
- miR-6826	Up	Plasma	49 Non responders, 41 responders	-	Microarray RTqPCR	miR-191	Predictive of vaccine efficacy	-	(178)
- miR-6875		Plasma	39 I-II, 57 III-IV	48 HD	RTqPCR	RNU6	Diagnosis Prognosis	0.842	(179)
- miR-23b	Down	Plasma	39 I-II, 57 III-IV	48 HD	RTqPCR	RNU6	Diagnosis Prognosis	0.842	(179)
- miR-1290	Up	Serum	45 I, 49 II, 55 III, 52 IV	57 HD	Microarray RTqPCR	Cel-miR-39	Staging Diagnosis Prognosis	0.83	(180)
- Panel with miR-31, miR-29c, miR-122, miR-192, miR-346, miR-372, miR-374c		Plasma	25 CRC 25 adenoma		Microfluidic array RTqPCR	RNU6 and miR-520d	Diagnosis	0.98	(171)

^aNumber in validation sets; ^bAUC for area under the receiver-operating characteristic curve.

advanced stages of the disease. Currently, lactate dehydrogenase (LDH) is the only AJCC circulating biomarker approved and used in metastatic disease but LDH has a very low specificity. Thus, the identification of efficient noninvasive biomarkers is necessary to improve early systemic melanoma recurrence and/or response to treatment. Circulating miRNAs related to melanoma remain less explored than those of other cancers. However, several studies demonstrating the potential interest of miRNA in melanoma were reviewed recently (182, 183) and the most recent are listed in Table 4. In 2011, the first study assessing the diagnostic role of the circulating miRNAs in melanoma conducted by Kanemaru et al. showed that the levels of miR-221, known to be increased in melanoma tissues, were increased in the serum of the metastatic patients and were correlated with tumor thickness. Thus the authors proposed that the serum level of miR-221 might be useful for diagnosis, staging, monitoring of the patients and prognosis (184). Then in 2013, for the first time, plasma levels of miR-21 were described to be elevated in melanoma and correlated to tumor mass (185). Usefulness of the panels of the circulating miRNAs in melanoma was also demonstrated such as a serum-based of 4 miRNA signature (miR-15b, miR-30d, miR-150 and miR-425) that predicts recurrence (186) or the 'MELmiR-7' panel that detects the presence of melanoma with a high sensitivity (93%) and a specificity ($\geq 82\%$) (187). Moreover the co-detection of miR-185 and miR-1246 in plasma allows an accurate discrimination of patients with metastatic melanoma from healthy individuals with a sensitivity of 90.5% and a specificity of 89.1% (188).

Ovarian cancer

Epithelial ovarian cancers (EOC), which account for 90% of ovarian cancers, are the leading cause of death among gynecological malignancies. The high mortality rate is due to the fact that this pathology is asymptomatic up to an advanced stage and therefore the diagnosis is most often too late. CA-125 is the most routinely used serum biomarker but is not sufficiently specific to diagnose EOC at an early stage. Indeed, CA-125 is only elevated in approximately 50% of stage I. New biomarkers for detecting early stage of EOC remain a major clinical challenge. About 20 studies, on circulating miRNAs, have been published in ovarian cancers (Table 5 and recent reviews, Refs. 193 and 194). Firstly they showed the diagnostic then the prognostic interest of circulating miRNAs. The first study identified a signature of eight exosomal miRNAs (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205

Table 4: Melanoma circulating miRNAs.

miRNA	Expression	Sample source	Patient cohort ^a	Healthy controls ^a	Type assay	Reference miRNA	Role	AUC ^b	Reference
- Panel with miR-15b, miR-30d, miR-150 and miR-425	Up	Serum	41 I, 20 II, 21 III		Microarray	miR-30c, miR-181a	Prognosis	0.79	(186)
- miR-210	Up	Plasma	148 III, 70 IV	41 HD	RTqPCR		Prognosis	0.623	(189)
- Panel with miR-122-5p and miR-3201	Up	Serum	32 0-I, 11 III, 9 IV	30 HD	qPCR array	Cel-miR-39	Prognosis	0.99 (healthy vs. SIII, 10 miRs), AUC = 0.97 (healthy vs. SIV, 5 miRs)	(190)
- Panel with miR-16, miR-211, miR-509-3p, miR-509-5p, miR-4487, miR-4706, miR-4731	Down	Serum	86 I, II, 50 III, 119 IV	130 HD	Taqman, Fluidigm	Cel-miR-39	Prognosis	0.9911	(187)
- miR-206	Down	Serum	20 I/II, 40 III/IV	30 HD	RTqPCR	RNU6	Prognosis	-	(191)
- miR-16	Down	Serum	30 I, 30 II, 30 III, 30 IV	120 HD	Microarray, RTqPCR	Cel-miR-39	Prognosis	0.779	(192)
- Panel with miR-1246 and miR-185		Plasma	73 IIIc, IV	64 HD	Microarray, RTqPCR	miR-103, miR-423-3p, miR-191	Diagnosis	0.95	(188)

^aNumber in validation sets; ^bAUC for area under the receiver-operating characteristic curve.

Table 5: Ovarian cancer circulating miRNAs.

miRNA	Expression	Sample source	Patient cohort ^a	Healthy controls ^a	Type assay	Reference miRNA	Role	AUC ^b	Reference
– Panel with miR-200b, let-7i, miR-122, miR-152-5p, miR-25-3p		Serum/plasma	25 serous carcinomas	25 HD	RTqPCR	miR-103a-3p, miR-27b-3p, miR-30b-5p and miR-101-3p	Diagnosis	No	(197)
– miR-200a	Up	Serum	70 epithelial ovarian cancer	70 HD	RTqPCR	RNU6	Prognosis	0.81 for miR-200a 0.833 for miR-200c	(198)
– miR-200b									
– miR-200c/b/c									
– miR-200c	Up	Serum	16 serous and 12 mucinous, 15 endometrial, 14 clear cells and 17 others	50 HD	RTqPCR		Prognosis	0.79 for miR-200c 0.75 for miR-141	(199)
– miR-141									
– miR-145	Down	Serum	18 serous, 12 mucinous, 24 endometrioid, 17 clear cell and 13 mixed	135	RTqPCR	RNU6	Prognosis	0.82	(200)
– miR-200b	Up	Plasma	33 serous		RTqPCR	miR-191	Prognosis	–	(201)
– miR-373, miR-200a, miR-200b and miR-200c	Up	Serum (exosome)	163 epithelial ovarian cancer	32 HD	RTqPCR	miR-484	Prognosis	0.925 for miR-200a/ b/c combination	(202)
– miR-1246	Up	Serum	Set 2: 58 serous ovarian carcinoma	65 HD	Microarray, RTqPCR, ddPCR		Diagnosis	0.893	(203)
– miR-125b	Up	Serum	135 epithelial ovarian cancer	54 benign ovarian tumor	Microarray, RTqPCR	miR-16	Diagnosis and prognosis	0.737	(204)

^aNumber in validation sets; ^bAUC for area under the receiver-operating characteristic curve.

and miR-214) in patients with ovarian cancer compared to benign disease (195). Another study, with the largest cohort of ovarian cancer patients, showed increase and decrease of expression of plasma miR-205 and let-7f, respectively, with a high diagnostic accuracy for EOC especially in patients with stage I disease (196). Moreover, a low rate of let-7f was correlated to poor progression-free survival (PFS) (196). The most representative circulating miRNAs in ovarian cancers are miR-21, miR-92, mi-93, miR-141, miR-200a, miR-200b, miR-200c and miR-205 (Table 5 and Refs. 193, 194).

Prostate cancer

Prostate cancer (PCa) is the most frequently diagnosed tumor in men. PSA (prostate-specific antigen) is the current gold standard biomarker for the diagnosis and response to the treatment of PCa. However, PSA has a low specificity with false positive results in patients with benign prostatic hyperplasia (BPH). Novel biomarkers are needed to distinguish between indolent and aggressive pathology and to reduce the risk of overdiagnosis and overtreatment. Many studies have highlighted the interest of circulating miRNAs in the diagnosis and prognosis of PCa. Recent reviews have been carried out on this subject (205–209). Moreover we listed the most recent publications in Table 6. The potential diagnostic of circulating miRNAs in PCa was first reported in 2008. Mitchell and coworkers found that serum miR-141 levels can distinguish PCa from healthy controls (5). Subsequently, several miRNA signatures were identified as accurate biomarkers in PCa, such as a panel of five miRNAs (miR-30c, miR-622, miR-1285, let-7c, let-7e) which discriminates PCa from BPH and from healthy controls with a very high accuracy area under curve (AUC of 0.924 and AUC of 0.860, respectively) (210) or a signature of 14 serum miRNAs to identify patients with a low risk of harboring aggressive PCa (211). However, despite the elevated number of studies in PCa, only three miRs were regularly reported: miR-141, miR-375 and miR-21 (Table 6 and refs. 205–209).

Circulating miRNAs analysis methods

A comprehensive overview of the circulating miRNAs studies unveils great differences in the results with a lack of concordance across the different projects (218, 219). This can partially be explained by methodological

heterogeneity that affects several steps of the miRNA analysis from the sample collection to the post-analytical steps. Below we will briefly present the main possible factors of the lack of concordance between the most miRNA signatures. Firstly, across the different studies, there is a discordance of the source of the circulating miRNAs such as serum or plasma, of the size of the patients and healthy control cohorts, of the preanalytical factors (such as delay before sample handling, centrifugation speed, storage temperature and time, freeze/thaw cycles, etc.) (90, 218, 220–222). Recent reports highlight the importance of proper and systematic sample collection, preparation and storage to avoid confounding variables influencing the results (223, 224). Both serum and plasma have been equally analyzed but might exhibit some differences in the miRNA profiles due in part to a release of miRNAs from blood cells or platelets during the coagulation process (90, 218, 221). Some precautions should also be taken with hemolysis that leads to contamination with red blood cell-enriched miRNAs such as miR-486-5p, miR-451, miR-92a, and miR-16. miRNA profiles which might be also susceptible to diurnal variations, fasting, hormonal changes, age of the donors, all parameters not often controlled in the various studies (218, 220, 221, 225, 226).

Others factors are more analytically related including RNA extraction method, measurement platforms, analysis and normalization of data. Several circulating miRNA isolation methods are available, phenol-based techniques associated or not with silica columns, and phenol-free techniques together with columns for RNA isolation (221). Differential efficiencies of these methods but with inconsistencies among the studies have been reported, depending notably on the different techniques of detection (225, 226). Some studies focus on the miRNAs contained within the extracellular vesicles such as exosomes. Many techniques are achievable to isolate vesicles in appreciable quantity and purity (227). The most common method uses differential ultracentrifugation but with several varied protocols (227). More recently polymer-based exosome precipitation solutions have been developed as a more rapid and simple method (227).

The accurate quantification of miRNA in bodily fluids is a difficult step due to their low quantity, their short sequence length, the high sequence conservation among family members, the wide range of miRNA concentration in body fluids and the high levels of interfering molecules measurement. Currently, several methods have emerged including hybridization-based approaches (like microarrays, nCounter Nanostring technology), reverse transcription quantitative PCR arrays (RT-qPCR)

Table 6: Prostate cancer circulating miRNAs.

miRNA	Expression	Sample source	Patient cohort ^a	Healthy controls ^a	Type assay	Reference miRNA	Role	AUC ^b	Reference
– miR-Score panel with let-7a, miR-24, miR-26b, miR-30c, miR-93, miR-100, miR-103, miR-106a, miR-107, miR-130b, miR-146a, miR-223, miR-451 and miR-874	Down	Serum	50 high grade, 50 low grade	50 benign hyperplasia	RTqPCR	Sp3, Sp6, cel-miR-39	Prognosis	'miR-Score' with negative predictive value of 0.939	(211)
– miR-375	Down	Plasma	59	16 benign hyperplasia	RTqPCR	RNU6	Diagnosis	0.809	(212)
– miR-141	Up	Serum/serum (exosome)	51	40	RTqPCR	Cel-miR-39	Diagnosis	0.869 (localized vs. metastatic)	(213)
– miR-410-5p	Up	Serum	149	121 benign hyperplasia and others urinary diseases, 57 HD	RTqPCR	RNU6	Diagnosis	0.8097	(214)
– miR-106a miR-130b miR-223		Plasma	36	31 benign hyperplasia	RTqPCR	miR-24	Diagnosis	0.81 for miR-106a/miR-130b	(215)
– Let-7c/e/i, miR-26a-5p, miR-26b-5p miR-18b-5p miR-25-3p	Down	Serum	64	60 benign hyperplasia	RTqPCR	miR-191-5p, miR-425-5p	Diagnosis and prognosis	0.77 for miR-106a/miR-223 0.667 for miR-18b-5p, miR-25-3p	(216)
– Pair with miR-297 and miR-19b-3p	Up	Plasma	18 metastatic, 18 non metastatic		Microarray		Diagnosis		(217)

^aNumber in validation sets; ^bAUC for area under the receiver-operating characteristic curve.

and next generation sequencing (NGS) (221, 225). The choice of measurement methods depends on the purpose of the project. RT-qPCR is the most used method because it is easily, quickly performed and has the best sensitivity, specificity, accuracy and reproducibility. Medium throughput profiling of miRNAs based on RTqPCR is possible with plates or microfluidic cards, using TaqMan® hydrolysis probes or locked-nucleic acid primers together with Sybr-green detection, the latter appearing to be more sensitive and specific (218, 222, 228). On the other hand, microarray based on RNA-DNA hybrid capture is used to perform an initial screening at lower cost while NGS technology allows the search for novel miRNAs or different isoforms (218, 222, 228). RT-qPCR is the gold standard technique for validation profiling microarray and NGS results (226).

Given all the variations in the source, the isolation and detection methods as mentioned above, the normalization of the raw data is a critical step to remove variations not related to the biological status (228–231). For large scale profiling data, a global mean or quantile normalization is commonly used (220, 228). But this method is not suitable for a limited number of miRNAs. Furthermore, to correct variability during the purification step and RTqPCR efficiency, standardization could be done through the use of synthetic spike-in miRNAs (such as cel-miR-39) but that does not take into account the differences of endogenous miRNA levels and release between samples. Following RTqPCR, two quantification strategies are used to determine the levels of miRNA. Firstly relative quantification measures the comparison of the expression levels of the target miRNA and of a reference gene, making the reference gene choice highly critical. Many reference genes have been used; the most described are *hsa-miR-16* or *RNU6* as endogenous genes and cel-miR-39 as exogenous gene (Tables 1–6). However, their choice has been controversial. Indeed miR-16 was shown to be released from hemolytic erythrocytes and several studies report that it is deregulated in cancer (218). RNU6, is a small nucleolar RNA, not an miRNA, therefore the efficiency of its extraction and amplification might be different. Some studies suggest that *RNA-U6* may be unsuitable as an endogenous reference gene (231–233). The use of multiple reference miRNAs instead of a single one is recommended in order to improve accuracy and limit the bias of the potential variation of the selected miRNA as it will provide statistically more significant results and will enable detection of small expression differences (231, 234–236). The selection of a set of stably expressed genes across the studies could be done using algorithms such as GeNorm or NormFinder.

The published reference gene combinations are multiple (Tables 1–6) but no specific combination emerges from the studies. The most efficient standardization method is the use of relative data normalization with endogenous and exogenous reference genes. Further studies are needed to identify universal miRNA references. Secondly, absolute quantification requires a standard curve for each miRNA analyzed from known concentrations of DNA standard molecules. This method is not optimal because it does not consider the influence of RNA quality. Droplet digital PCR (ddPCR) appears as a novel alternative method providing the advantages of absolute quantification without a reference standard curve or an endogenous control.

Conclusion

Since their discovery in 2008, as described here, there is a plethora of publications assessing the use of circulating miRNAs as biomarkers in oncology. Despite the great enthusiasm for their potential in clinical application, currently the circulating miRNA measurement has not yet gone into clinical practice. There is not yet any individual or panel miRNA validated as a biomarker for cancer disease. In fact as shown in the different reviews as well as in Table 1, very few overlaps could be observed across relatively similar studies. Many miRNAs are reported in only one publication. Discordant results are sometimes published such as for miRNA described upregulated or downregulated. Some miRNAs used as reference genes in some publications are shown to be differentially expressed in other publications (miR-16, miR-103, etc.). Moreover, to date, the large majority of studies examined only a limited number of samples. Precautions have to be taken about the cohort composition since correlations of miRNA levels with age, sex and ethnicity have been demonstrated.

Thus before clinical application of the measurement of circulating miRNAs as biomarkers in oncology, several issues have to be overcome. Large-scale inter-laboratory studies have to be performed. New advances in standardization of all the steps in the process of miRNA analysis are required to improve knowledge on these new biomarkers such as choice of the biofluid, limiting contamination from cellular elements, standardization of the preanalytic and analytic methods, choice of a reference gene and normalization method. Overcoming all these challenges is urgently needed to render the promising circulating miRNAs as reliable and sensitive biomarkers from the bench to the bedside.

List of abbreviations

AUC	area under curve
BPH	benign prostatic hyperplasia
EOC	epithelial ovarian cancer
LDH	lactate dehydrogenase
miRNA	microRNAs
NSCLC	non-small cell lung cancer
PCa	Prostate cancer
ROC	receiver operating characteristic curve.

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