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miRNA profiling of cancer

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Abstract

A steadily growing number of studies have shown that microRNAs have key roles in the regulation of cellular processes and that their dysregulation is essential to keep the malignant phenotype of cancer cells. The distorted and unique expression profile of microRNAs in different types and subsets of tumor coupled with their presence in biological fluids make of microRNAs an attractive source of sensitive biomarkers. Here, we will discuss how microRNA profiles are altered in cancer, highlighting their potential as sensitive biomarkers for cancer risk stratification, outcome prediction and classification of histological subtypes. We will also evaluate the current knowledge on the use of microRNAs as circulating biomarkers, hoping that further studies will lead to the application of microRNA signature in prognostic and predictive markers that can improve patient health.

Introduction

In the last decade non-coding RNAs have emerged as a new class of key regulators involved in development, normal physiology, and many different types of disease. MicroRNAs (miRNAs) represent the major class of small endogenous non-coding RNAs and control almost one-third of all human genes [1–2]. MiRNAs are single stranded RNAs of 19–25 nucleotides in length that negatively regulate gene expression by base-pairing to partially complementary sites on the target messenger RNAs (mRNAs), usually in the 3' untranslated region (UTR) [3]. Binding of a miRNA to the target mRNA typically leads to translational repression and exonucleolytic mRNA decay, although highly complementary targets can be cleaved endonucleolytically [4]. As data accumulated proposing fundamental roles for miRNAs in proliferation, differentiation, survival and apoptosis, it is not surprising that miRNAs were found to be important in tumorigenesis and considered promising therapeutic targets for novel cancer treatments [5]. After our initial discovery that miR-15/16 cluster is deleted or downregulated in patients with chronic lymphocytic leukemia [6•], myriad reports established that neoplastic tissues had a cancer-associated miRNA signature. This miRNA alteration allows the accurate classification of the different malignancies and the identification of the tissue of origin for poorly differentiated tumors. The cause of the widespread differential expression of miRNA genes between malignant and normal cells can be explained by different mechanisms including **a)** chromosomal alterations of the miRNA genes, **b)** DNA point mutations, **c)** epigenetic mechanisms or **d)** alterations in the machinery responsible for miRNA production (Figure 1) [7]. Similar to the coding genes, miRNAs can be either over- or under-expressed and they can act as tumor-suppressors or oncogenes

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based on the downstream target that the miRNA controls. miR-15 and miR-16 were the first described tumor-suppressor miRNAs and their loss, by releasing the inhibition upon tumor-promoting genes, such as BCL2, BMI1, CCND2 and CCND1, promotes cell growth and tumor progression [6•, 8–9•,10]. Alternatively, miR-21 is highly up-regulated in the majority of cancer tissues and by repressing pro-apoptotic genes, such as PTEN or PDCD4, stimulates proliferation and tumor initiation [11–12••]. The development of different high-throughput miRNA profiling technologies (Table 1) has allowed the characterization of the miRNA expression profile for several malignancies including chronic lymphocytic leukemia [13], breast cancer [14], lung cancer [15], thyroid papillary carcinoma [16], pancreatic tumors [17], glioblastoma [18], gastric cancer [19], prostate cancer [20], hepatocellular carcinoma [21]. Surprisingly, the use of miRNA expression is newly becoming highly preferred to the traditional gene expression profiles for a variety of reasons. First, the remarkable stability of miRNAs, due to their short length, has allowed scientists to perform analyses also in samples considered to be technically challenging, such as formalin fixed specimens. Additionally, highly sensitive and refined miRNA detection techniques provide high reliability in the use of miRNAs as a diagnostic tools. Finally, miRNA expression has demonstrated the ability to identify the tissue of origin for cancer that have already spread in multiple metastatic sites, thereby reducing patient's psychological burden and overall procedure costs [22•, 23]. In the following sections, we will discuss some of the more important approaches that have been taken to identify cancer-associated miRNA expression profiles and their potential use in clinical application.

miRNAs as a cancer biomarkers

A large number of miRNA genome-wide studies in different tumors have highlighted that selective groups of distinct miRNAs - miRNA fingerprints - are commonly dysregulated in specific types of human malignancies and often associated with diagnosis, staging, progression, prognosis and response to clinical therapies. These results have been confirmed over time in different cohorts of patients and provided the evidence that miRNA dysregulation in cancer is unlikely a random event (Table 2). In 2006, our laboratory published the first comprehensive profile of miRNAs in cancer by analyzing miRNA expression in 540 samples including 363 solid tumors from the six most common malignancies (breast, prostate, lung, stomach, pancreas, thyroid) and 177 normal tissues [24••]. A common “miRNoma” in cancer was identified consisting of 36 overexpressed and 21 downregulated miRNAs: among them, some of the well characterized cancer associated miRs, such as miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155 (Table 2). Golub and coworkers also showed that miRNA profiles of tumor tissues paralleled the developmental origins of the malignant tissues, supporting the idea that miRNA expression patterns encode the developmental history of human cancers [25••]. For example, tumors of epithelial origin presented a different miRNA expression from haematopoietic malignancies. Furthermore, distinct patterns of miRNA expression can be observed within a single developmental lineage and reflect mechanisms of transformation. For example, miRNA profiles can subgroup acute lymphoblastic leukaemia (ALL) specimens into three major groups: one containing all t(9;22) BCR/ABL- and t(12;21) TEL/AML1-positive samples; a second group containing T-cell ALL samples; and a third group containing the MLL gene rearrangement. Thereafter, Rosenfeld and coworkers identified, by using miRNA microarray data of 253 samples, a transparent classifier based on 48 miRNAs that predicts with high confidence and accuracy the tissue from which cancers of unknown primary origin arose [22•]. Specifically, the classification accuracy reached 100% for most tissue classes analyzed, including 131 metastatic samples and an independent blinded test-set of 83 samples, demonstrating the great diagnostic value of miRNAs [22•].

One of the most important aspect of the miRNA fingerprints in cancer is their discriminatory role for different cancer subtypes or correlation with specific neoplastic events, such as oncogenic activation. This characteristic provides additional information for the prognosis and treatment of cancer when combined with standard gene profiling. Numerous studies have shown that miRNAs can classify breast cancers into a specific tumor pathological phenotype (i.e., Estrogen Receptor and Progesterone Receptor status, proliferation, tumor stage, metastatic state, HER2 status) [14, 26], as well as the molecular subtype (Luminal A, Luminal B, Basal-like, HER2+ and Normal-like) [27]. To date, miR-200 family is positively associated with a well-differentiated breast cancer phenotype (luminal tumors) and it is very low expressed in the poorly differentiated claudin-low subtype of breast cancer [28]. In fact, it has been shown that miR-200 family members directly target ZEB1 and ZEB2, two transcription factors that control the epithelial-to-mesenchymal (EMT) transition, essential for cancer cells to become motile, invasive, and survive the metastatic journey [29, 30]. While miR-200 family expression is high in the luminal breast cancer subtype, miR-221/222 cluster is overexpressed in triple negative breast cancers, particularly those that have undergone EMT [31,32]. Increasing miR-221 or -222 can affect various characteristics associated with EMT, including increased invasive capacity, and anoikis resistance [33]. Inhibition of miR-221/222 in basal-like cancer cells promoted mesenchymal to epithelial transition (MET) in part by directly targeting trichorhinophalangeal 1 (TRPS1), a transcriptional repressor of ZEB2 that can promote MET [33]. Furthermore, a recent publication from our laboratory showed that miR-221 is down-regulated in ductal carcinoma in situ and highly up-regulated in invasive ductal carcinoma [34], thus revealing a possible role for miR-222 and miR-221 cluster in the development of highly proliferative and aggressive breast cancer.

Another important feature of miRNA in cancer is their predictive role for the mutational status of important clinical biomarkers. The first association came from our laboratory in 2005, when we reported a 13 miRNA signature predictive of the ZAP70 and IgV_H status in CLL patients [12]. Similarly, low expression of *miR-193a*, *miR-338*, and *miR-565* was associated with melanomas carrying a *BRAF* missense mutation at the commonly involved residue V600E [35]. Interestingly, miR-193 was also reported to be downregulated in *BRAFV600E* mutated-thyroid cancer cell lines compared with normal thyroid tissue, suggesting that *miR-193a* may have an important and common role in *BRAF*-associated tumorigenesis. Likewise, up-regulation of miR-155 in acute myeloid leukemia was strongly but independently predictive of FLT3 gene mutations, which have been associated with patients that have increased risk of relapse [36]. miR155 is one of the most intensively studied miRNAs, not least of all because of the relatively early realization that it is abnormally expressed in specific forms of lymphomas, including Hodgkin lymphoma (HL), diffuse large B-cell (DLBCL) and primary mediastinal B cell lymphomas, but not primary Burkitt lymphoma [37–39]. miR-155 over-expression in mouse resulted in pre-B-cell expansion and bone marrow replacement, splenomegaly, and lymphopenia that preceded the development of lymphoblastic leukemia and lymphoma [40].

miRNAs have been also linked to the prediction of specific cytogenetic abnormalities that have prognostic implications and are present in the majority of hematological malignancies, such as CLL. Specifically, 32 miRNAs discriminated between CLL patients with the 17p and 11q deletions, who experience the aggressive form of the disease, and CLL patients with the 13q deletion or normal cytogenetic profiles, who experience the indolent form [41]. Another example of deregulated miRNA expression associated to cytogenetic abnormalities is represented by the miRNAs identified in multiple myeloma samples that are associated with the most common IgH translocations (t(4;14) and t(11;14)) and del(13q) [42]. Calin et al. have reported that more than 50% of miRNAs are located at genomic sites that are disrupted or amplified in various cancers [43]. For example, the 13q31 region, which is

amplified in DLBCL [44], mantle cell [45], and follicular [46] B-cell lymphomas, harbors a cluster of seven miRNAs, the miR-17/92 cluster [47]. In agreement, this miRNA cluster was found to be increased ~10-fold in 65% of B-cell lymphoma samples [48]. When this cluster is overexpressed with the *myc* oncogene, it greatly accelerated *c-myc*-induced pre-B cell lymphomas in mice and reduced apoptosis [48]. Another example of relation between cytogenetic abnormalities and microRNAs is represented by the t(11;14)(q13;q32) translocation and miR-16 [10]. This translocation represents a genetic hallmark of mantle cell lymphoma that displaces the *CCND1* gene on chromosome 11 downstream to the enhancer region of the IgH gene on chromosome 14 and causes its overexpression. Studies of patient samples have shown that truncations exist within the *CCND1* mRNA 3' UTR and the truncation leads to a worse prognosis. Since miR-16 represses *CCND1*, the truncation of the 3' UTR reported in lymphoma cell lines prevents proper miR-16 repression of *CCND1* mRNA, resulting in increased proliferation and shorter survival.

Since the large contribution of miRNAs in tumorigenesis, it is not surprising that miRNA expression can be also predictive of clinical survival and response to chemotherapy. To this regard, low miR-191 and high miR-193a were associated with a significant reduced survival in melanoma patients [49]. In CLL, high levels of miR-15/16 cluster are associated to a good prognosis, according with their tumor-suppressor activity demonstrated *in vitro* and *in vivo* [8, 13]. In lung cancer, a large miRNA genome-wide expression analyses showed that the levels of both miR-155 and let-7a-2 are associated with poor survival [15]. The potential prognostic value of let-7 in lung cancer was also shown in an independent study of Japanese patients where repression of let-7 correlated with significantly shorter survival time after curative surgery and stage of the disease [50]. Another study reported that liver cancer patients whose tumors had low miR-26 expression had shorter overall survival but a better response to interferon therapy [51]. In gastric cancer a robust 7-miRNAs signature predicts outcome [52]. In various cancer, miR-21 is an index of poor outcome [53–55] and it is also an important predictor of response to chemotherapy: high miR-21 expression levels are predictive of response to gemcitabine in pancreatic cancer patients [56]. Of note, Medina et al. demonstrated that miR-21 is a bona fide oncogenic miRNA [12••]. Induction of miR-21 in mouse resulted in spontaneous development of lymphoma that most closely resembled precursor B-cell lymphoblastic lymphoma/leukemia. Remarkably, repression of the miR-21 levels in mice with significant tumor loads resulted in complete disappearance of the tumors within 1 week.

miRNAs as new serum biomarkers

Current diagnostic techniques, whether based on more traditional approaches, such as histological evaluation, or based on a more personalized approach, such as Oncotype DX (Genomic Health, Inc., CA, USA) or Mammaprint (Agendia, Amsterdam, The Netherlands), both essentially rely on the direct sampling of the tumor material. Although direct measurements of biomarkers within tissues have greatly improved diagnosis and survivability, the invasive and unpleasant nature of the diagnostic procedures limits their application for most clinical conditions. In this regard, many researchers worldwide have made numerous efforts in the identification of new biomarkers in body fluids (serum, plasma and urine) due to their ease of collection and the fact that they reflect a particular physiological or pathological state. Many studies in fact have shown that specific cancer characteristics, both genetics and epigenetics, are detectable in the plasma and serum of cancer patients and can be useful in diagnostic to monitor ongoing pathological processes, such as alpha-fetoprotein (AFP) for liver cancer [57], prostate-specific antigen (PSA) for prostate cancer [58], carcinoembryonic antigen (CEA) and (CA125) for breast and ovarian cancer, respectively [59–60]. In 2007, small RNAs, including miRNAs, were identified in total RNAs preparation derived from biological fluid [61••]. The authors showed that

exosomes from mast cells, natural vesicles secreted by a variety of cells, contain mature miRNAs which can be transferred to another cell and can be functional in this new recipient. Shortly afterwards, Lawrie et al. published the first report of miRs modulation in serum of B-cell lymphoma patients and that high miR-21 serum levels were associated with increased relapsed-free survival but not with overall survival [62]. In 2008, Tewari group showed that miRs are present in human plasma as a stable molecules and provided direct evidence that tumor-derived miRNAs can enter the circulation even when originating from an epithelial cancer type [63]. Recently, the global relationship between tissue miRNAs and circulating miRNAs in normal population has been investigated and showed that the composition of liver miRNAs correlates most closely with the miRNAs found in blood, suggesting that under normal conditions the liver may have the most contact with circulating blood [64]. Conversely, placenta, testis, and brain exhibit the weakest correlations between tissue miRNA populations and those found in blood [64]. Although reports indicate that circulating miRNAs are secreted or leaked from normal or tumor tissues, the mechanisms of the miRNA release and their function into circulation remain largely unknown. Even if many questions still remain unanswered, an immediate interest emerged for the investigation of miRs as non-invasive biomarkers in circulating blood, and a large list of new blood-derived miRs profiles emerged (Table 3). The most striking finding is the general upmodulation of miR-21 and/or miR-155 as a putative diagnostic and prognostic markers in several different malignancies. To date, high levels of circulating miR-21 were predictive of the estrogen receptor negative status while high levels of miR-155 were associated to a positive progesterone receptor status in breast cancer [65–66]. A comprehensive analysis of miRNA profiles in serum has been performed by Chen and coworkers [67]. The authors showed that miRNAs are abundant in the majority of body fluids (serum, plasma, amniotic fluid, urine, tears, ascetic fluid) and, by using Solexa deep sequencing, they identified 190 known miRs in the serum of healthy donors. They also determined that patients with lung cancer, diabetes and colorectal cancer have a significantly different expression of miRs in their serum compared to healthy subject. Specifically, miR-25 and miR-223, highly expressed in lung tumors, were also enriched in patient serum and their expression predicted lung cancer in an independent set of 75 healthy donors and 152 cancer patients. Recently, our group provided the first evidence for the roles of circulating miRNAs in cancer by demonstrating that tumor-secreted miRs are able to interact with the Toll-like receptors of immune cells to stimulate the production of prometastatic inflammatory cytokines and inducing the pro-tumor inflammatory processes [68]. In this scenario, circulating miRNAs can act as signals for receptor activation, a function that is completely independent of their conventional role in post-transcriptional gene regulation.

Conclusion

There is no doubt that miRNAs are involved in tumorigenesis and regulate the development and progression of human malignancies. Although many progresses have been made in the understanding of miRNA involvement in cancer, many questions still need to be answered before translating miRNA profiling into clinical practice. One of the biggest challenges for the future years will be the creation of cancer specific miRNA signatures that can be highly reproducible and independently predictive of clinico-biological features of the tumor to improve diagnosis and treatment.

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Figure 1. miRNA dysregulation in cancer

Dysregulation of miRs expression in cancer compared to the normal tissues of origin is a general phenomenon that has been largely characterized in almost all neoplasia. Global repression of miRNAs expression in cancer cells is believed to induce an undifferentiated phenotype. Indeed the increase of specific miRs, the "oncomiRs", confers aggressiveness and resistance to cell death. In the figure, we depicted the main processes involved in miRNA dysregulation, such as **a)** chromosomal alterations of the miRNA genes, **b)** DNA point mutations, **c)** epigenetic mechanisms or **d)** alterations in the machinery responsible for miRNA production. **e)** In addition to their intracellular functions, recent studies have demonstrated that miRNAs can be released or leaked from cancer cells and circulated in a remarkably stable form within blood. Many studies have demonstrated the circulating miRNA levels correlate significantly with cancer progression, therapeutic response, and patient survival. As RISC-associated, microvesicles-related or as a free miRNAs or pre-miRNAs, the function of circulatory miRNAs is largely unknown. Many questions are still missing an answer: are circulating miRNAs a result of leakage from cancer cells or active release? Besides bio-markers, can miRNA drive or activate molecules that help the body defend against cancer? **f)** Representation of the miRNA modulation in the neoplastic tissues compared to normal tissues of origin: an "oncomiR" upmodulation and a "tumor-suppressor miR" repression are shown in the two graphics.

Table 1

microRNA profiling technologies

technologies	advantages	disadvantages	Assay or platform/Vendor	cost
quantitative reverse transcription PCR (qRT-PCR)	Highly sensitivity and specificity. Low amount of RNA template and useful for absolute quantification	Useful only for known miRs. Relative low number of samples that can be processed per day.	TaqMan individual assays (ABI) miRCURY LNA qPCR (Exiqon) TaqMan OpenArray (ABI) TaqMan TLDA microfluidics card (ABI) Biomark HD system (Fluidigm) SmartChip human microRNA (Wafergen) miScript miRNA PCR array (SABiosciences/Qiagen)	\$\$
miRNA microarray	Relative low cost and high- throughput with respect to the number of samples that can be processed per day	Lower specificity than qRT-PCR or RNA sequencing. Cannot be use for new miRs	Geniom Biochip miRNA (CBC febit) GeneChip miRNA array (Affymetrix) GenoExplorer (Genosensor) MicroRNA microarray (Agilent) miRCURY LNA microRNA array (Exiqon) NCode miRNA array (Invitrogen) nCounter (Nanostring) OneArray (Phalanx Biotech) Sentrix array matrix and BeadChips (Illumina) μParaFlo biochip array (LC Biosciences)	\$
RNA sequencing: high-throughput	Useful for the detection of novel miRs. High sensitivity in discriminating between very similar miRs	High cost. Large computational work for data analyses	HiSeq 2000 and Genome Analyzer IIX (Illumina) Solid (ABI) GS FLX+ 454 sequencing (Roche)	\$\$\$
RNA sequencing: smaller scale	Useful for the detection of novel miRs. High sensitivity in discriminating between very similar miRs	High cost. Large computational work for data analyses	Ion Torrent (Invitrogen) MiSeq (Illumina) GS Junior(454) (Roche)	\$\$\$

Table 2

microRNA dysregulated in cancer

microRNA	genomic location	expression in cancer	function	mechanism of deregulation	targets
let-7a-2	11q24	down in breast, lung, colon, ovarian and stomach cancer	tumor-suppressor	repressed by MYC	KRAS, HMGA2, MYC, DICER, BCL-XL, IMP-1, CDC34, IL6
miR-15/-16	13q31	down in CLL, prostate cancer and pituitary adenomas	tumor-suppressor	genomic loss, mutated, activated by p53	BCL2, COX2, CHECK1, CCNE1, CCND1, CCND2, BMI-1, FGF2, FGFR1, VEGF, VEGFR2, CDC25a
miR-29 family	7q32 1q30	down in AML, CLL, lung and breast cancer, lymphoma, hepatocarcinoma, rhabdomyosarcoma	tumor-suppressor	genomic loss, activated by p53, repressed by MYC	CDK6, MCL1, TCL1, DNMT1, DNMT3a, DNMT3b
miR-34 family	1p36 11q23	Down in colon, lung, breast, kidney and bladder cancer	tumor-suppressor	repressed by MYC	SIRT1, BCL2, NOTCH, HMGA2, MYC, MET, AXL, NANOG, SOX2, MYCN, SNAIL
miR-26a	3p22	down in liver cancer	tumor-suppressor	repressed by MYC	CCND2, CCNE2
miR-200 family	1p36 12p13	down in aggressive breast cancer	tumor-suppressor	repressed by ZEB1/2;	ZEB1, ZEB2, BMI-1, SUZ-12, FN1, LEPR, CTNNB1, JAG1, MALM2, MALM3, p38alpha
miR-155	21q21	up in high risk CLL, AML, breast, lung, colon cancer and lymphoma	oncogene	activated by NF-KB	SOC31, BACH1, MEIS1, ETS1, FOXO3A, hMSH2, hMSH6, hMLH1, SMAD5, WEE1, SHIP1, CEBPB
miR-21	17q23	up in lung, breast, pancreas stomach, prostate, CLL, AML, glioblastoma, myeloma	oncogene	activated by IL6, GFI1alpha	PTEN, TPM1, PDCD4, SPRY1, TIMP3, RECK
miR-221/-222	Xp11	up in invasive ductal carcinoma, lung cancer, hepatocellular carcinoma, papillary thyroid cancer	oncogene	activated by MET in lung cancer; repressed by ERalpha in breast; activated by PLZF in melanoma; activated by NF-Kb and cJun in prostate cancer and glioblastoma cells	p27(Kip1), p57(Kip2), PTEN, TIMP3, FOXO3A, ERalpha, KIT, TRSP1, DICER, APAF1, PUMA, PTPμ
miR-17/92	13q22	up in lung, breast, colon,	oncogene	activated by E2F1 and MYC	PTEN, BIM, HIF1, PTPRO, p63, E2F2, E2F3, TSP-1, CTGF, p21(WAF1), JAK1, SMAD4, TGFbetaII, Page 21 of 22 MnSOD, GPX2, TRXR2

Table 3

microRNA profile in blood

Cancer type	biological fluid	modulated miRs	prognostic value
HNSCC	plasma	miR-184	-
Breast cancer	serum	miR-34, -10b, -155	all three miRs showed a correlation with metastasis
	serum		miR-155 associated to PR status
	serum	miR-21, -106a, -155; miR-126, -199a, -335	
	whole blood	miR-195	miR-21, -10b associated to ER-; let-7a reduced in lymph node metastasis
NSCLC	serum	mir-25, -223	-
	exosome from plasma	miR-17, -21, -106a, -146, -155, -191, -192, -203, -205, -210, -212, -214	-
	serum	miR-486, -30d; miR-1, -499	correlated with overall survival
	serum	miR-10b, -155	miR-10b is correlated with lymph node metastasis
Colorectal cancer	plasma	miR-221	associated with poor overall survival
Hepatocellular carcinoma	serum	miR-25, -375, let-7f	-
	serum	miR-21, -122, -223	-
Pancreatic carcinoma	plasma	miR-21	-
	plasma	miR-21, -155, -196a	-
Prostate cancer	serum	miR-141	-
	serum	miR-20b, -874, -1274, -1207, -93, -106a, miR-24, -223, -26b, -30c	miR-24 is correlated to metastasis
	serum	miR-21	miR-21 is associated to docetaxel resistance