MicroRNAs in clinical oncology: at the crossroads between promises and problems

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MicroRNAs in clinical oncology: at the crossroads between promises and problems

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ABSTRACT

MicroRNAs (miRNAs) are small RNAs that do not code for proteins, but function by controlling protein expression of other genes. miRNAs have been shown to control cell growth, differentiation and apoptosis. Shortly after their discovery, miRNAs have been found to be associated with cancer. Earlier reports have shown that human cancers frequently show a distorted expression profile of miRNAs. In this review, the biogenesis of miRNAs and potential mechanisms of their dysregulation and involvement in cancer pathogenesis are discussed. The current literature on potential applications of miRNAs in the field of clinical oncology from diagnostic to prognostic and predictive applications at the tissue, and more recently, serum levels, is reviewed. The potential therapeutic applications of miRNAs and RNAi in the field of cancer are summarised. Finally, some of the potential challenges that face the transition of miRNAs from a research setting into a clinical application are highlighted, with a future prospective of the incorporation of miRNAs in cancer patient management.

MicroRNAs (miRNAs) are small RNAs that have lengths ranging from 18 to 25 nucleotides and do not code for proteins.[1] More than 12 years ago, Ambros *et al* discovered a non-coding 22 nucleotide segment of RNA transcribed from the lin-4 gene in the nematode *Caenohabditis elegans*. This nucleotide segment was shown to interact with the 3′ untranslated region of lin-14, subsequently leading to a reduced expression level of the later.[2] Although these small RNA segments played a major role in gene regulation by binding to other RNA segments, they have gone undetected by geneticists for more than 30 years. The short length of these miRNAs was the main reason why they have slipped undetected. Today, miRNAs play a significant role in opening a new realm of research that extends past the nematode to include all multicellular organisms that experience regulated gene expression[3] and will undoubtedly lead to significant advances, especially in cancer research.[4]

This review summarises current evidence of the involvement of miRNAs in cancer and the spectrum of potential clinical applications of miRNAs in human cancer that encompasses diagnosis, prognosis, prediction of treatment efficiency and therapy. We also discuss the challenges that face the transition of these applications from the research setting to clinical services.

DEFINITIONS

In addition to mRNA, several classes of small non-coding RNAs have been identified. These include miRNAs, small interfering RNA (siRNA), rRNA, tRNA and small nuclear RNA (snRNA). The distinction between miRNA and siRNA cannot be made based only on the structure or the mechanism of action.[5] Typically, for a molecule to be classified as an miRNA, it has to meet two of the following three criteria: (i) its expression should be confirmed by Northern blotting, RT-PCR or RNase protection assay (expression criterion); (ii) the sequence should be present in one arm of the hairpin precursor, which lacks large internal loops or bulges (structure criterion); (iii) the sequences should be phylogenetically conserved (conservation criterion).[6] The most important distinguishing criteria between miRNA and siRNA have been recently described[6 7] and are summarised in table 1.

BIOGENESIS AND PHYSIOLOGICAL FUNCTIONS

Biogenesis of miRNAs has been discussed in detail in a number of recent excellent reviews.[1 8 9] Beginning with miRNA gene transcription by RNA polymerase II, a long primary miRNA is produced. This is termed "pri-miRNA". This is then modified in the nucleus through capping, polyadenylation and subsequently cleaving into smaller segments by an RNase III enzyme "Drosha". A hairpin precursor of 60–70 nucleotides is formed, and is termed "pre-miRNA", which is then transported to the cytoplasm by "exportin 5" where it is modified by another RNase enzyme "Dicer". The resulting miRNA is a mature 19–24 nucleotide duplex. One of the two mature miRNA strands is integrated into a large protein complex called RNA-induced silencing complex (RISC); that then cleaves the target mRNA or represses its translation to protein, depending on the degree of complementarity with the target mRNA. The other strand is subsequently destroyed via cleavage or a bypass mechanism.

miRNAs have a number of biologically diverse functions.[3] By binding through imprecise sequence complementarities, miRNAs control the expression of thousands of genes,[3 5] which subsequently influence several cell regulation pathways, including cell mobility, differentiation, development, proliferation and apoptosis (for more details, please refer to recent review articles[10–14]). miRNAs have only been found in multicellular organisms. This suggests that miRNAs are essential towards cell maturation; either by differentiation into specific cell types or maintaining cells at a differentiation stage.[3]

miRNAs AND CANCER

Shortly after their discovery, miRNAs have been found to be associated with cancer.[15] This link is

Table 1 The major distinguishing features between microRNA (miRNA) and small interfering RNA (siRNA)

	miRNA	siRNA
1	Always endogenous	Can be endogenous or exogenous
2	Usually derived from genomic loci distinct from recognised genes	Usually derived from mRNAs, transposons, viruses or heterochromatic DNA
3	Formed from local RNA hairpin structures	Produced from long exogenous or endogenous dsRNA molecules (very long hairpins or bio-molecular duplexes)
4	A single miRNA:miRNA* duplex is generated from each hairpin precursor	Multiple duplexes are generated
5	Highly conserved among species	Rarely conserved
6	Hetero-silencing; ie, target remote loci	Auto-silencing; ie, target the same locus
7	Processed in the nucleus by Drosha and in the cytoplasm by dicer	Processed in the cytoplasm by dicer
8	Perform function by either suppressing protein synthesis or mRNA cleavage	Perform function by mRNA cleavage

inspired by viewing cancer as rapid and abnormal cell growth, migration and apoptosis. Earlier reports have proven that human cancers frequently show a distorted expression profile of miRNAs. miRNA dysregulation has been reported in many types of tumours, eg lymphoma, colorectal cancer, breast cancer, glioblastoma, lung cancer and testicular germ cell cancer, among many others.[16 17]

A further link to carcinogenesis stems from the finding that more than half of the cancer-dysregulated miRNAs are located in cancer hotspot chromosomal regions, such as fragile sites, regions of loss of heterozygosity, amplification or common breakpoint regions.[18] One example of this is miR-15 and miR-16 genes whose expression is often suppressed in chronic lymphocytic leukaemia; they are found in a chromosomal region that is usually deleted in this malignancy.[19]

As fig 1 shows, there are different possible mechanisms for altered miRNA gene expression in cancer. For more detailed discussion on this topic, we refer the reader to a number of recent excellent reviews.[20–23] miRNAs can be located in areas that undergo genetic alterations (like deletions or amplifications) in specific cancers. Germ-line or somatic mutations of miRNAs (or their targets) can also lead to altered function. Recent evidence has also shown that epigenetic changes (eg, methylation) can also affect miRNA expression in malignancy.[24] Other potential mechanisms include defective post-transcriptional regulation; it is found that miRNA expression is suppressed in many cancers due to a functional defect of the miRNA processing enzyme Drosha.[23]

MiRNAs can be either causes or effects of the carcinogenic process. As fig 2 illustrates, miRNAs down-regulate gene expression, and fluctuations in their concentration can affect either oncogenes or tumour suppressor genes. In this regard, miRNAs can be viewed as having oncogenic (eg, miR-21, miR-106a and miR-155) or tumour-suppressor (eg, let-7, miR-15a/16, miR-34a and miR-143/145) effects depending on their targets.[25] In other words, over-expressed miRNAs in cancers may function as oncogenes by negatively regulating tumour suppressor genes and/or genes that control cell differentiation or apoptosis, or might function as tumour suppressor genes by suppressing oncogenes.[26] On the other hand, some miRNAs can be downstream targets of oncogenes or tumour suppressor genes, like p53.[27]

There are three possible venues that can explain the link between miRNAs and cancer progression: cell adhesion, angiogenesis, and cell matrix digestion and signalling.[28] Few miRNAs, like mir-9, have been found to regulate E-cadherin expression. Loss of E-cadherin is an adverse prognostic factor in a wide variety of gastrointestinal, endocrine, pulmonary and genitourinary carcinomas as well as melanoma. Also, a number of miRNAs have been proposed to regulate the expression of vascular endothelial growth factors, hypoxia-related genes and angiopoetins. Finally, a series of miRNAs have been linked to matrix metalloproteases (MMPs), MMP inhibitors (or TIMPS), and plasminogen-related proteases associated with tumour invasion of stroma.[28]

In addition to the direct effect on cancer cells, one important factor to consider is the altered tumour–host interactions leading to sustained angiogenesis and the ability to invade and metastasise. As discussed in more details in a recent review,[29] it is possible that miRNAs may regulate these aspects of tumour biology, including cell adhesion, neovascularisation and tissue invasion.

CLINICAL APPLICATIONS

The spectrum of potential applications of miRNAs in cancer is broad and continues to grow. They can be used for diagnosis, tumour classification, prognosis and prediction of treatment efficiency, in addition to therapeutic applications in many human malignancies.[30]

miRNAs as diagnostic markers

Accumulating reports highlight the potential diagnostic utility of miRNA in cancer.[8 16 31] As table 2 shows, diagnostic applications have three aspects; miRNA expression profiles can be used: to distinguish normal from malignant tissues; to identify the tissue of origin in poorly differentiated tumours or tumours of unknown origin; and finally, to distinguish the different subtypes of the same tumour.

Earlier reports have shown the presence of tissue-specific signatures of miRNA expression. This is potentially helpful in the categorisation of tumours that otherwise cannot be properly classified by morphology and immunohistochemistry alone, an important problem in surgical pathology practice. Interestingly, Lu *et al* observed a superior ability of miRNAs in classifying tumours compared to mRNA profiling.[16] Very recently, reports have shown that miRNAs can be also detected in serum in a remarkably stable form.[32–34] One study showed that miRNAs originating from human prostate cancer can access the circulation directly, not only inside exfoliated cells.[34] Another study identified specific expression patterns of serum miRNAs for lung cancer, colorectal cancer and diabetes.[32] If confirmed by independent studies, these results open the promise of utilising miRNAs for the blood-based detection of cancer.

Increasing evidence has shown that miRNA expression is useful in classifying a variety of cancers into molecular subtypes with different outcomes. As table 2 shows, thyroid and breast cancers are two good examples.

It is worth mentioning here that Mattie *et al* showed that miRNA expression profiling can be performed on small clinical samples, such as breast and prostate cancer biopsy specimens.[35]

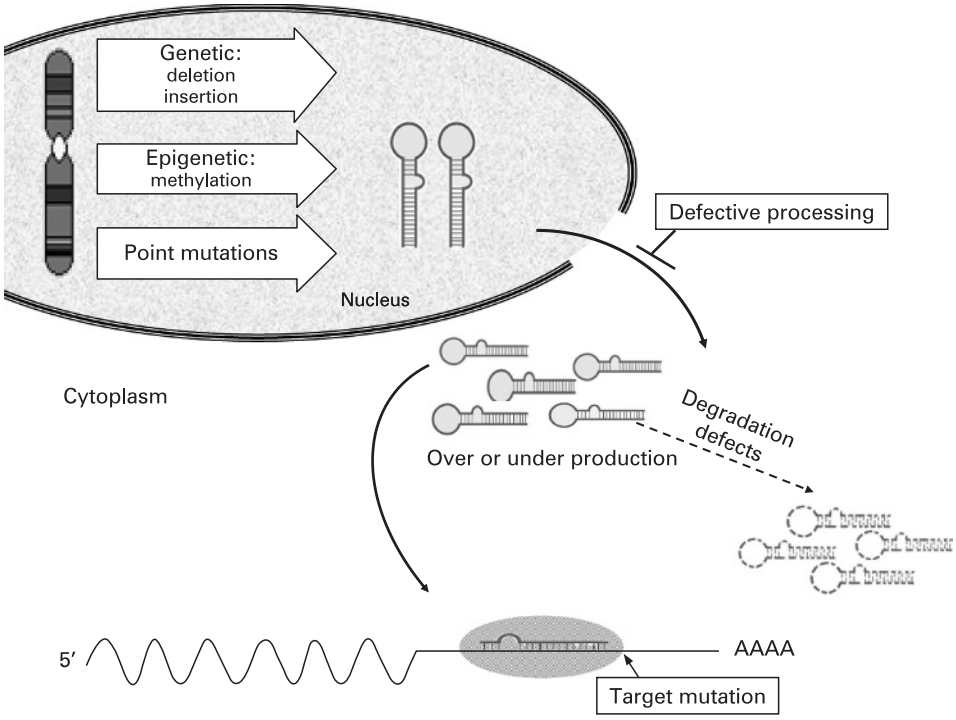

Figure 1 A schematic presentation of the possible mechanisms of altered microRNA (miRNA) expression in cancer. miRNAs can be located in areas that undergo genetic alterations (eg, deletions or amplifications) in cancer. Mutations of miRNAs or their targets, either germ-line or somatic can also lead to altered function. Epigenetic changes (eg, methylation) can also affect miRNA expression in malignancy. Other potential mechanisms include defective post-transcriptional regulation, including processing or degradation.

Other clinical applications

The clinical utilities of miRNAs in cancer extend beyond diagnostics to other important potential applications. As table 3 shows, a series of studies published during the last few years have revealed the association of miRNA expression with clinical outcome and prognosis in a variety of human tumours.[30] Certain germ-line mutations were found to occur in cases with familial cancer. In addition, preliminary evidence suggests that miRNAs can serve as predictive markers and indicators of disease relapse and also metastasis.

It is important to mention that conclusions obtained from studies performed in cell lines and a limited number of clinical samples can be very different from those derived from studies performed in a large number of human tumour specimens. In a recent study by Gee *et al*,[36] it was found that the expression of miR10b does not correlate with distant metastasis or poor prognosis in breast cancer, whereas Ma *et al* have found that miR10b overexpression is correlated with metastatic behaviour.[37]

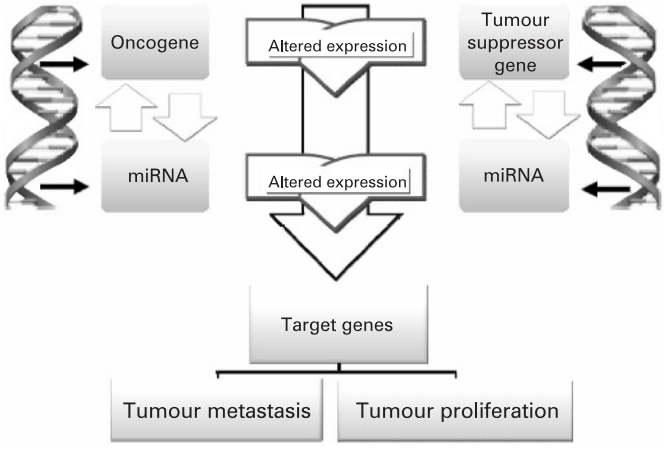

Figure 2 A schematic showing the possible mechanisms of microRNA (miRNA) involvement in carcinogenesis. Altered miRNA expression may result from gross chromosomal alterations (eg, insertions or deletions) or over- or under-expression of upstream control molecules, like oncogenes or tumour suppressor genes. miRNA dysregulation affects target genes that are critical to tumour initiation and/or progression. miRNAs can also affect the expression levels of certain oncogenes or tumour suppressor genes whose targets are keys for carcinogenesis.

Potential therapeutic applications of miRNAs and siRNAs

Recently, short interfering RNAs (siRNAs) and miRNAs have gained considerable attention as potential promising targets for cancer therapeutics. The fact that miRNA dysregulation in cancer has a pathogenic effect provides the rationale for using miRNAs as potential therapeutic targets in cancer.[38] miRNA-based cancer gene therapy offers the theoretical appeal of targeting multiple gene networks that are controlled by a single, aberrantly expressed miRNA.[25] Reconstitution of tumour-suppressor miRNAs, or sequence-specific knockdown of oncogenic miRNAs has produced favourable anti-tumour outcomes in experimental models; these are now being tested for therapeutic applications in different cancers.[4]

There are different available strategies to modulate miRNA effects in cancer for therapeutic purposes.[39] Inhibition of miRNA expression can be achieved using anti-sense oligonucleotides "antagomirs", or their chemically modified versions, like locked nucleic acids (LNA),[40] or inhibiting production of the mature forms by affecting their processing. Restoring miRNA expression, on the other hand, can be achieved by transfecting miRNA mimics either directly or through vectors.

Table 2 Examples of the diagnostic applications of miRNAs in different malignancies

Cancer type	Study material	Clinical utility	Reference
Breast	Normal vs. cancer tissues	► 29 miRNAs significantly deregulated in cancer vs normal ► 15 miRNAs can distinguish normal from tumour with 100% accuracy ► miRNA expression correlated with specific biopathological features, such as oestrogen and progesterone receptor expression	59
Pancreas	Normal, chronic pancreatitis and ductal carcinoma tissues	► 25 miRNAs correctly differentiated pancreatic cancer from benign pancreas in 90% of samples ► 23 miRNAs differentiated pancreatic cancer from chronic pancreatitis with 93% accuracy	60
Kidney	Normal vs. cancer tissues	► 33 miRNAs dysregulated in cancer	Our unpublished data
Pooled cancers	Different cancer types	► miRNA expression profiles classify poorly differentiated tumours ► miRNA profiles reflect the developmental lineage of the tumours	16
Prostate	Serum of normal vs. cancer	► miRNAs are stable in serum ► miR-141 can distinguish prostate cancer from healthy controls	34
Thyroid	Different cancer subtypes	► miR-146b is a potential adjunct marker of papillary thyroid carcinoma	61
Thyroid	Different cancer subtypes	► miRNA expression profile differentiates ATC from normal thyroid tissues and from PTC	62
Prostate	Normal, benign and pre-malignant tissues	► miRNAs separated metastatic prostate cancers from normal and non-malignant precursors	35
Breast	Different cancer subtypes	► miRNAs distinguished tumours according to oestrogen receptor and HER2/neu subtypes	35

ATC, anaplastic thyroid carcinoma; PTC, papillary thyroid carcinoma.

Corsten *et al* showed, by using cytotoxic agents in conjunction with the suppression of miR-21 promotes, a synergistic rise in caspase activity and a significant decrease in cell viability in human glioma cells within both in vitro and in vivo brain tumour mouse xenograft.[41] As an initial step to help directing miRNA research towards therapeutic applications, Rossi *et al* attempted to provide an updated catalogue of miRNAs located at fragile sites or at cancer susceptibility loci.[42]

One of the first demonstrations of inhibition of expression of a constitutively over-expressed tumour protein by synthetic miRNA targeting 3′ un-translated regions of its mRNA was done for the HER-2 proto-oncogene.[43] Scott *et al* have also recently explored the potential of exogenously applied miRNAs to suppress ERBB oncogene family proteins.[44] In haematological malignancies, the potential of therapeutic applications of miRNA-based technology has been recently reviewed.[45] Another study has shown that tandem arrays of miRNA mimics can effectively alter the leukaemogenic potency.[46] Also, re-expression of miR-203 has been shown to have therapeutic benefits in specific haematopoietic malignancies.[47]

Let-7 administration was found to reduce tumour formation in vivo in the lungs of animals expressing a G12D activating mutation for the K-ras oncogenes.[48] In another recent study, miRNA-mediated knockdown of survivin in combination with apoptin over-expression significantly induced apoptosis and inhibited cell growth. This combined strategy offers potential advantages in control of tumourigenesis.[49] In hepatocellular carcinoma, specific and powerful gene silencing that can be achieved by activating RNAi has generated enthusiasm for exploiting this pathway for therapy. Many studies have been carried out with the aim of silencing hepatocellular carcinoma-related cellular oncogenes or the hepatocarcinogenic hepatitis B virus (HBV) and hepatitis C virus (HCV). Proof of principle studies has demonstrated promising results, and an early clinical trial assessing RNAi-based HBV therapy is currently in progress.[50] In brain tumours new therapeutic strategies involving miRNA silencing or miRNA mimics could be proposed based on the roles of these small non-coding RNAs as oncogenes and tumour suppressors.[43]

There are, however, some important issues which need to be resolved prior to the consideration of any miRNA-based experimental cancer gene therapy, such as the possibility for non-specific immune activation and the lack of a defined, optimal mode of delivery systems. Prior to any applications of miRNAs in clinical trials there is an urgent need for definitive mRNA target validation and a complete understanding of

Table 3 Potential clinical applications of microRNAs (miRNAs) in cancer patient management

Cancer type	Application	Summary of results	Reference
Pancreas	Prognosis	► In node-positive disease, six miRNAs can distinguish long from short term survivors ► High expression of *miR-196a-2* predicts poor survival	60
CLL	Prognosis	► miRNAs are associated with zap-70 expression ► Predictors of early progression	63
Lung	Prognosis	► Differential miRNA expression correlates with poor survival	64
Lung	Prognosis	► Overexpression of miR-21 is an independent negative prognostic factor in NSCLC	65
Cholangiocarcinoma	Therapy	► Alterations in miRNA expression contribute to tumour growth and response to chemotherapy	66
Hepatocellular carcinoma	Prognosis	► A set of 19 miRNAs significantly correlated with disease outcome	67
Uterine leiomyoma	Race susceptibility	► A specific miRNA signature associated with race	68
Breast	Prediction of treatment efficiency	► miRNAs as predictive tamoxifen-resistant breast cancer markers	69
Rectum	Prediction of treatment efficiency	► miRNAs are associated with response to therapy	70
Lung	Cancer susceptibility	► A variant allele in a KRAS miRNA complementary site is significantly associated with increased risk for NSCLC	71
CLL	Cancer susceptibility	► Mutations in miR-15A and miR-16-1 in CLL patients ► Mutations found in 73% of patients with family history of cancer	72
Breast	Metastasis susceptibility	► Tumour metastasis initiated by miRNA-10b	37

CLL, chronic lymphocytic leukaemia; NSCLC, non-small cell lung cancer.

rate-limiting cellular components that impact the efficiency of this post-transcriptional gene-silencing phenomenon.[25] A major focus in this area has been on target gene-specific siRNA-delivery technology in vivo. In particular, creating a pinpoint delivery system for siRNAs is a priority because such a system would be a key technology for the development of the next generation of drugs, including anticancer therapies.[51] Recent reports on locked nucleic acid (LNA) mediated miRNA silencing in rodents and primates support the potential of LNA-modified oligonucleotides in studying miRNA functions in vivo and in the future development of miRNA-based therapeutics.[52] Epigenetic intervention strategies that could be used to amend defects in miRNA/mRNA interactions have also been recently reviewed.[53]

FROM BENCH TO BEDSIDE: IMPORTANT CHALLENGES

The transition of miRNA applications from the research setting to the clinical stage necessitates addressing several challenges that have been discussed in recent reviews.[54 55] These include the need to identify all miRNAs and their targets, technical issues and the need for standardisation, and defining the specific clinical questions that need to be addressed.

Identification of all human miRNAs is a key issue for a better understanding of the role of miRNAs and their potential applications in cancer. So far, about 700 miRNAs have been identified in the human genome, and an estimated similar number is expected to be discovered.[56] The next step, which is more challenging, is the identification and the experimental validation of miRNA targets. Reports have shown that each miRNA can target multiple genes, and that the same mRNA can be targeted by multiple miRNAs. A number of target prediction programs are currently available including PicTar, miRanda and TargetScan.[57] The inherent difficulty of target prediction stems from the imperfect complementarities between the miRNA and its target, leading to minimal consistency between different prediction programs, and making experimental validation extremely important.

Another important challenge that needs to be addressed is standardisation of miRNA analysis. Several techniques and platforms already exist including microarray analysis, bead analysis, and quantitative RT-PCR. Standardisation also includes the type of miRNA to be analysed (mature versus pre-miRNAs) and the number of miRNAs to be included in a clinical test (screening all known miRNAs versus a selected pattern of highly informative miRNAs). Uniformity of the experimental condition is also required. Standardisation also includes the type of specimen to be analysed (fresh frozen tissue versus formalin fixed paraffin-embedded tissue) and the appropriate method of specimen storage in addition to uniform interpretation of test results. Heterogeneity of the tumour is another important factor to be considered. Tumour tissue represents a mixture of tumour, adjacent normal and stromal elements. Other technical issues include test sensitivity (especially with low abundant miRNAs) and specificity (especially with members of the same family).

A critical question that needs to be answered is the ability of miRNAs to provide additional information for patient diagnosis and management that goes beyond the classical factors that are currently available. A major limitation of most published reports so far is the heterogeneity of the analysed material, from tissues to cell lines and combining different histological types, stages and grades. Unfortunately, there is a lack of prospective studies that can accurately define the significance of miRNA testing. An advantage documented by recent reports is the ability to perform miRNA testing on paraffin-embedded tissues.[58] This will significantly accelerate the validation of the clinical utility of miRNA in malignancy.

Moreover, many of the experiments at the discovery phase lack statistical significance since they are not initially designed with enough power to address the hypothesis to be examined. Added to this, is the lack of well defined clinically annotated cases. The results of such experiments should therefore be validated, preferably by an independent study.

Take-home messages

- MicroRNAs (miRNAs) represent an emerging new tool for cancer patient management, with a wide range of potential clinical applications, including diagnosis, assessment of prognosis and prediction of treatment efficiency.
- They also have potential therapeutic applications.
- More effort is still needed to allow the transition of miRNAs from being a research tool to clinical use.

A FUTURE PROSPECT

Although we are in the early phases of miRNA research, it is anticipated that miRNAs will have a significant impact in improving the patient's diagnosis and management. With more transparent accumulation of reports about the clinical significance of miRNAs, it is likely that they will have a diagnostic role in many cancers. In addition, miRNA analysis of the tissue material from cancer patients is likely to provide additional significant contribution that aids to the individualisation of treatment through developing a ''tumour fingerprint'' with specific information about aggressiveness, and treatment options for each individual patient. Two major milestones in this field are the recently reported stability of miRNAs in serum[34] and the ability to perform molecular miRNA profiling from a needle core biopsy.[35] If these are confirmed by multiple reports, a new strategy of utilising miRNA profiling as an adjutant diagnostic or prognostic tool without compromising the clinical diagnosis is opened up.

Finally, it is unlikely that miRNA analysis will replace the existing tools for tumour diagnosis and management. A more practical view is that they will be slowly added in conjunction with our existing tools.

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