MicroRNAs in the cancer clinic

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1. ABSTRACT

Over recent years there have been major advances in our understanding of tumour biology which have led to improved diagnostic and prognostic techniques and the development of novel targeted therapies. However the reliability of such biomarkers is questionable and the efficacy of new treatments remains predominantly limited by a combination of drug resistance, toxicity and persisting insufficiencies in our comprehension of tumour-signalling pathways. Following their recent discovery, microRNAs (miRNAs) have been established as key regulators of gene expression, and their putative roles as oncogenes and tumour suppressor genes has provided a potentially new dimension to our clinical approach to cancer diagnosis and treatment. Their role as biomarkers and therapeutic targets is appealing but several obstacles have as yet limited our ability to translate this potential into a clinical reality. This review focuses on currently accepted roles of miRNAs in cancer pathogenesis, and highlights the challenges and breakthroughs in this field to date with relevance to the cancer clinic.

2. INTRODUCTION

MiRNAs constitute an evolutionarily conserved class of pleiotropically acting small non-coding RNAs that suppress gene expression post-transcriptionally via sequence-specific interactions with the 3’ untranslated regions (UTRs) of cognate messenger-RNA (mRNA) targets (1). These interactions result in either inhibition of translation of targeted mRNAs or their degradation. An individual miRNA can regulate many specific mRNAs and together >1400 human miRNAs potentially modulate over one-third of the mRNA species encoded in the genome, thereby controlling essential biological systems including cell survival and growth (2). Over 50% of miRNA-encoding loci reside in chromosomal regions altered by tumorigenesis (3) and a number of miRNAs function as classical oncogenes or tumor suppressor genes (4). The first study to demonstrate a link between miRNAs and cancer identified both miR-15a and miR-16-1 were downregulated or absent in most patients with B-cell chronic lymphocytic leukemia, which often resulted from a deletion at 13q14 where the genes encoding these miRNAs are located (5).
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Table 1. miRNAs implicated in the pathogenesis of human malignancies

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>miRNA</th>
<th>Expression in pathological state</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>miR-155</td>
<td>Overexpressed</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>miR-21</td>
<td>Overexpressed</td>
<td>(64)</td>
</tr>
<tr>
<td></td>
<td>miR-17-92</td>
<td>Overexpressed</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>miR-9</td>
<td>Overexpressed</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>miR-31</td>
<td>Downregulated</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>let-7</td>
<td>Downregulated</td>
<td>(66)</td>
</tr>
<tr>
<td></td>
<td>miR-126</td>
<td>Downregulated</td>
<td>(23)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>miR-155</td>
<td>Overexpressed</td>
<td>(67)</td>
</tr>
<tr>
<td></td>
<td>miR-21</td>
<td>Overexpressed</td>
<td>(68)</td>
</tr>
<tr>
<td></td>
<td>miR-34</td>
<td>Downregulated</td>
<td>(69)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>miR-21</td>
<td>Overexpressed</td>
<td>(70)</td>
</tr>
<tr>
<td></td>
<td>miR-17-92</td>
<td>Overexpressed</td>
<td>(71)</td>
</tr>
<tr>
<td></td>
<td>miR-155</td>
<td>Overexpressed</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td>let-7</td>
<td>Downregulated</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td>miR-1</td>
<td>Downregulated</td>
<td>(72)</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>miR-221</td>
<td>Overexpressed</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td>miR-21</td>
<td>Overexpressed</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td>miR-151</td>
<td>Overexpressed</td>
<td>(75)</td>
</tr>
<tr>
<td></td>
<td>miR-26a</td>
<td>Downregulated</td>
<td>(29)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>miR-21</td>
<td>Overexpressed</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td>miR-34a</td>
<td>Overexpressed</td>
<td>(77)</td>
</tr>
<tr>
<td></td>
<td>miR-15a/16-1</td>
<td>Overexpressed</td>
<td>(78)</td>
</tr>
<tr>
<td></td>
<td>miR-143</td>
<td>Downregulated</td>
<td>(79)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>miR-21</td>
<td>Overexpressed</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>miR-34</td>
<td>Downregulated</td>
<td>(80)</td>
</tr>
<tr>
<td></td>
<td>let-7</td>
<td>Downregulated</td>
<td>(81)</td>
</tr>
<tr>
<td>AML</td>
<td>miR-29a</td>
<td>Overexpressed</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td>miR-10a/b</td>
<td>Overexpressed</td>
<td>(83)</td>
</tr>
<tr>
<td></td>
<td>miR-29b</td>
<td>Downregulated</td>
<td>(84)</td>
</tr>
<tr>
<td>CLL</td>
<td>miR-135</td>
<td>Overexpressed</td>
<td>(85)</td>
</tr>
<tr>
<td></td>
<td>miR-15a/16-1</td>
<td>Overexpressed</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td>miR-34a</td>
<td>Downregulated</td>
<td>(86)</td>
</tr>
</tbody>
</table>

Extensive tumor profiling studies have implicated many miRNAs in the development, progression and metastasis of many tumour types (Table 1). Their discovery goes someway to explaining the gap that frequently exists between tumour genotype and phenotype, and has furthered our understanding of post-transcriptional regulation of gene expression in which they play a critical role. The functional importance of miRNAs in both healthy and pathological states has exposed their notable potential as disease biomarkers and therapeutic targets, which forms the focus of this review.

3. miRNAs AS BIOMARKERS

3.1. Diagnostic biomarkers

Cancers are often diagnosed at a late stage, with associated poor prognosis. The oncogenic and tumor suppressive nature of miRNAs, and the discovery of tumour-specific miRNA signatures suggests a potentially important role for these molecules as early diagnostic biomarkers. In a study of 104 matched pairs of primary malignant and non-malignant lung tissue, Yanaihara et al. (6) identified a group of 43 differentially expressed miRNAs that could successfully discriminate between the two groups. Gee et al. (7) found a panel of miRNAs (including the miR-200 family) that were down-regulated in malignant pleural mesothelioma compared to lung adenocarcinoma, and could be used consistently to distinguish between these two tumors (7). A tissue profiling study demonstrated a role for miRNAs in distinguishing primary brain tumours from secondary metastases originating from distant sites (8) and other studies have identified panels of potential diagnostic biomarkers in hepatocellular carcinoma (9), breast carcinoma (10) and pancreatic endocrine and acinar tumours (11).

Current diagnostic methods are usually invasive and technically challenging, indicating a substantial need for novel non-invasive biomarkers for early tumor detection. The ability to profile miRNAs in the circulation represents a less invasive method of investigating disease-specific miRNAs and is a promising alternative approach to tumor tissue profiling techniques. An essential requirement for developing circulating miRNA-based diagnostics is the ability to accurately isolate and measure miRNA species. Chen et al. (12) was one of the first studies to demonstrate the presence of miRNAs in human serum and plasma. Using small RNA deep sequencing they identified approximately 100 circulating miRNAs in healthy Chinese subjects and subsequently studied specific expression patterns of serum miRNAs in lung and colorectal cancer patients, comparing them to healthy subjects. In lung cancer patients, 28 miRNAs were absent and 63 new miRNA species were detected, and in the colorectal cohort 69 serum miRNAs were detected that were not present in the healthy cohort, which included miR-221, previously shown to be increased in colorectal tumour specimens (13). Interestingly, despite the high concentration of RNases in plasma and serum, circulating miRNAs were resistant to RNase A digestion.

Evidence for the role of secretory miRNAs as diagnostic tools is growing. Elevated plasma miR-155, miR-197, and miR-182 levels accurately discriminated lung cancer patients from healthy controls (81.33% sensitivity and 86.76% specificity). miR-155 and miR-197 levels were higher in patients with metastasis than those without, and were significantly decreased in responsive patients during chemotherapy (14). Shen et al. (15) demonstrated that patients with malignant solitary pulmonary nodules had elevated plasma levels of miR-21 and miR-210 but lower miR-486-5p levels compared to those with benign lesions or healthy controls. A comparison of plasma miRNA expression levels between 20 early breast cancer patients and 20 healthy controls identified 31 differentially expressed miRNAs in Caucasian patients and 18 in African American patients (16). Liu et al. (17) used logistic modeling to show plasma miR-16 and miR-196a levels could discriminate pancreatic cancer from chronic pancreatitis and normal controls. This was even more sensitive with the inclusion of serum CA19-9 in the logistic model, and was even effective at identifying stage I disease.

Despite this growing evidence, the use of plasma miRNAs as diagnostic tools in clinical practice remains sparse. Although there have been advancements in isolation techniques and despite the apparent stability of circulating miRNAs, their measurement may be confounded by variability in the levels of cellular miRNAs from hematological origins and from circulating tumor cells. To truly gain a greater understanding of the relevance of free circulating miRNA levels, fractionation processes must be
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employed to differentiate between those derived from tumor tissue and circulating cells. This should improve the sensitivity of these methods, as microdissection has in tissue-based miRNA biomarker studies. Furthermore, miRNA levels in other bodily fluids may play a future role in cancer diagnosis. Studies have identified miRNAs as potential diagnostic biomarkers in human bile (18), sputum (19) and faeces (20) although further studies are required to validate this.

3.2. Prognostic biomarkers

miRNA profiling studies have demonstrated a number of correlations between differentially expressed miRNAs and prognosis in various tumour types. In lung adenocarcinoma, low let-7a and elevated miR-155 expression was associated with poor survival (6), and miR-137 and miR-372 levels correlated with increased risk of relapse and worse survival (21). Overexpression of miR-155 and miR-21 correlated with poor outcome in early-brest cancer and dysregulated expression of miR-9, miR-10b, miR-21 and miR-315 was associated with an increased risk of metastasis (10) (22) (23) (24). Schetter et al. (25) (26) demonstrated in two independent cohorts that elevated miR-21 levels correlated with poor survival in colorectal cancer, and the same miRNA has been implicated in predicting survival in localised pancreatic cancer, as has miR-196a-2 (27). In hepatocellular carcinoma decreased miR-122 (28) and miR-26 (29) correlated with poor patient survival and miR-151-5p with an increased risk of intrahepatic metastasis (30). Historically, factors such as tumor grade, size and lymph node involvement have been used in early stage disease to determine treatment strategies, however there is sufficient evidence to suggest that miRNAs would serve as useful adjuncts or even alternatives to such methods.

3.3. Predictive biomarkers

Despite the development of novel targeted anti-cancer therapies, chemotherapy represents the foundation for treatment regimens for most hematological and solid malignancies. However, resistance of tumor cells to chemotherapy, and to a lesser extent targeted therapies, remains a major obstacle to effective treatment. Recent studies postulate that aberrant miRNA expression might be involved in tumor resistance to current therapies. This suggests a role for miRNAs as predictive biomarkers, and that modulation of tumor miRNAs may be exploited to improve treatment response in addition to producing direct anti-tumor effects.

3.3.1. Breast Cancer

miR-155 knockdown increased sensitivity of breast cancer cells to chemotherapy through regulation of FOXO3a (31), and down-regulation of miR-21 augmented breast cancer cell response to taxol (32). Multidrug-resistance in specific MCF-7 cell lines is associated with reduced levels of miR-326 (33) and miR-451 (34) leading to upregulated expression of the multi-drug resistance-associated protein 1 (MRP-1/ABCC1) and the multi-drug resistance 1 protein (MDR1) respectively. Reconstitution of miR-451 expression sensitised cells to doxorubicin.

Furthermore, the mir-17-92 and mir-106a-363 families have been shown to control estrogen receptor-alpha transcription and cellular responses to estrogen in breast cancer and their expression levels may predict response to antiestrogens (35).

3.3.2. Pancreatic Cancer

Inhibition of miR-21 increased sensitivity of pancreatic adenocarcinoma cells to gemcitabine (36). Two clinical studies provide further evidence that miR-21 affects chemosensitivity in pancreatic cancer. The first study using 81 pancreatic ductal adenocarcinoma (PDAC) samples from patients treated with gemcitabine, found high miR-21 expression was associated with poorer overall survival in both the adjuvant and metastatic settings (37), and a subsequent study demonstrated a correlation between low miR-21 expression and improved outcome (disease-free and overall survival) in patients with localised PDAC treated with adjuvant gemcitabine or 5-fluorouracil chemotherapy (38).

3.3.3. Ovarian Cancer

Cisplatin resistance has been linked to miR-214 overexpression via targeting of PTEN (39). Li et al. (40) demonstrated an association between taxol-resistance in human ovarian cancer cells lines and increased expression of MDR1/P-glycoprotein due to down-regulation of miR-27a, and transfection with pre-miR-27a re-sensitized these cells to taxol. Furthermore, in a study of 37 stage III ovarian cancer patients, seven miRNAs, including miR-27a, were significantly differentially expressed in tumors from platinum-resistant versus –sensitive patients. High miR-27a expression was associated with a particularly poor prognosis in terms of OS (41).

3.3.4. Chronic lymphocytic leukemia (CLL)

Response to fludarabine therapy in CLL is associated with differential miRNA expression. In a study of fludarabine-treated CLL patients, Ferracin et al. (42) identified 37 miRNAs that distinguished responders from non-responders, with miR-21, miR-148a and miR-122 being more highly expressed in non-responding patients. In a similar study involving 50 CLL patients, fludarabine resistance was associated with decreased miR-29a and increased miR-181a expression (43).

3.3.5. Hepatocellular Carcinoma

The let-7 family targets Bcl-Xl in hepatocellular carcinoma cell lines, and overexpression of these miRNAs increased sorafenib-induced apoptosis in cell culture experiments (44). Furthermore, Ji et al. (29) demonstrated an association between improved response and low miR-26 expression profiling in over 200 HCC patients treated with interferon-α.

Although these data are promising, larger prospective trials are required to validate the role of miRNAs as predictive biomarkers, but such studies may lead to significant changes in treatment algorithms for certain tumour types.
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4. miRNA-MODULATING AGENTS AS CANCER THERAPEUTICS

4.1. Theory versus reality

A greater understanding of miRNA expression and function, and the growing evidence that miRNA deregulation is involved in cancer development and progression supports their role as potential therapeutic targets in cancer. Down-regulation of target oncogenes by re-expression of tumor suppressor miRNAs, or repression of tumor suppressor genes by silencing an oncomir could impair tumour growth and metastasis.

A single miRNA can target many mRNAs suggesting that miRNA-modulating therapy could simultaneously modify a number of relevant gene networks within a tumour, leading to significant biological effects on phenotype. Accordingly, a new class of drugs that specifically target small RNA pathways via replacement of tumor suppressive miRNAs with synthetic or viral vector encoded miRNA mimics or antisense-mediated inhibition of oncogenic miRNAs are currently in development. However, a number of concerns must be addressed before such therapies can be safely applied to the clinic. A miRNA introduced into a tumor cell may target mRNAs other than that/those intended, although utilizing a number of miRNAs at lower concentrations to target a single miRNA may enhance the specificity of silencing, and selecting a miRNA which targets multiple genes within a pathway may consolidate silencing and reduce these undesired effects (45).

The development of approaches to deliver miRNA-modulating agents to target tissues is also a major difficulty. Barriers to systemic delivery include degradation by serum and tissue nucleases, failure to cross the capillary endothelium due to size, uptake by scavenger macrophages and ineffective endocytosis and endosomal release in target cells (46). Additionally, intracellular RNA-binding proteins may further limit the activity of miRNA-modulating agents within target cells. The strategies employed to modulate miRNA activity for therapeutic purposes and measures utilised to overcome potential obstacles are discussed below.

4.2. DELIVERY SYSTEMS

4.2.1. miRNA inhibition

miRNA antagonists must selectively hybridize with their endogenous miRNA target via partial or complete complementarity, thereby preventing interactions between the miRNA and its target mRNA (47). The most basic examples are anti-miRNA oligonucleotides (AMOs) which consist of a ‘naked’ single-stranded molecule that inhibits miRNAs via complementary binding. An early example of their use in vivo demonstrated that intravenous (i.v.) injection of an AMO into mice silenced hepatocyte expression of Fas and protected against fulminant hepatitis (48).

However ‘naked’ oligomers are relatively unstable and are easily degraded by endogenous RNases, resulting in their limited efficacy by systemic administration. To overcome this, oligomers can be modified by the addition of cholesterol conjugated 2′-O-methyl groups to produce more stable ‘antagomirs’. Krutzfeldt et al. (49) demonstrated that a single i.v. injection of an antagomir designed to target cholesterol-regulating miR-122 in mice, resulted in prolonged miR-122 silencing in the liver and a significant decrease in serum cholesterol levels. Ma et al. (50) showed the systemic delivery of an antagomir to miR-10b in a mouse mammary tumour model prevented metastasis formation. Furthermore, a single intratumoral injection of antagomir-221/222 into Me665/1 melanoma xenografts in nude mice, significantly inhibited tumour progression for one week with no documented toxicity (51), and intraperitoneal (i.p.) injection of antagomir-182 reduced hepatic metastasis of melanoma cells in a mouse model (52).

Further adaptations led to the development of ‘locked nucleic acid’ (LNA) oligomers. Such oligonucleotides contain a ribose moiety that is functionally locked into a C3′-endo conformation via the addition of a methylene bridge, that confers greater stability, increased miRNA-binding affinity and lower toxicity (47). LNA antisense oligomers to miR-122 have been shown to reduce serum cholesterol levels in healthy and obese mice as well as healthy non-human primates. miR-122 is also essential for Hepatitis C virus (HCV) RNA replication and systemic delivery of an LNA antisense oligomer to miR-122 (SPC3649, Santaris Pharma) in HCV-infected chimpanzees led to prolonged 300-fold suppression in HCV viremia (53). In a subsequent Phase I single-dose safety study in humans, SPC3649 demonstrated limited toxicity and a clear dose-dependent pharmacology and has now entered into Phase II, making it the first miRNA-modulating therapy to reach this stage (54). Such agents are yet to show similar success in the cancer setting.

Vector-encoded RNA molecules, termed ‘miRNA sponges’, represent a novel approach to miRNA-modulating therapy. Containing multiple partially complementary 3′UTR binding sites, they competitively bind to miRNAs thus liberating their mRNA targets. They can be designed to carry a number of different binding sites, enabling simultaneous inhibition of multiple members of a miRNA cluster or different miRNAs acting on the same target. This is an advantage over ASOs which only target single miRNAs. Furthermore they can be stably integrated into the genome, enabling the development of transgenic animals and stable cell lines that are functionally deficient in certain miRNAs. Valastyan et al. (24) orthoimplanted MCF7-Ras cells expressing a sponge vector targeting the anti-metastatic miR-31 into mice, resulting in a significant induction of lung metastases. Gentner et al. (55) demonstrated that expression of an anti-miR-223 vector in hematopoietic stem cells, resulted in the functional knockdown of miR-223 when these cells were transplanted into lethally irradiated mice. However, a number of factors make miRNA sponges unsuitable for therapeutic use in humans and therefore no such approaches have been trialed yet. Firstly there is the risk of insertional mutagenesis in target
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cells, and vector size and poor biodistribution limits their systemic use. The future design of small-molecule drugs targeting miRNAs (SMIRs) may overcome this.

4.2.2. miRNA replacement/mimics

Although studies directed at inhibiting oncogenic miRNAs have shown promise, the restoration of tumour suppressive miRNAs using miRNA replacement or mimics may be a more efficacious, less toxic strategy. Developing a miRNA mimic requires the design of structures able to interact with the RNA-inducible silencing complex (RISC) and target the same miRNA as the endogenous miRNA. Such approaches have utilised chemically modified miRNAs, viral vectors and nanoparticle delivery systems in pre-clinical, and more recently, clinical models.

Chemical adaptations to miRNAs mimics have, as with miRNA antagonists, allowed more effective administration to their target tissue. Takeshita et al. (56) used tail vein injections to administer a chemically modified miR-16 precursor (a tumor suppressor known to be downregulated in prostate cancer) or ‘scrambled mimic’ to a murine model of bony metastatic prostate cancer. The miRNA was complexed with atelocollagen, which promoted uptake into the bone metastases and was effectively and persistently detected in target tissue for >3 days. Growth of the bone metastases was significantly lower in miR-16-treated mice than those administered the ‘scrambled mimic’.

Adeno-associated viruses (AAVs) allow the persistent transcription and expression of miRNAs at high levels in target tissues with a low risk of insertional mutagenesis compared to other viral delivery systems (57). Downregulation of miR-26a is associated with HCC and tail vein delivery of a miR-26a-expressing AAV into a murine HCC model suppressed tumorigenesis (58). Furthermore, although approximately 90% of hepatocytes were transduced with miR-26a in this model, there were no signs of hepatotoxicity or dysregulation of endogenously expressed miRNAs (58). Another murine model utilised the intranasal instillation of an adenosine encoding let-7 or a negative control (n.c.) miRNA, and the cre recombinase in transgenic K-RAS G12D mice (which induces expression of the K-RAS mutant G12D and the formation lung tumors). Following surgical removal of the lungs, histology revealed mice that received cre/let-7 developed far fewer and smaller tumors than those that received cre/n.c., further establishing a role for let-7 as a tumor suppressor (59). A previous study had shown that the intratumoral injection of let-7 directly into murine non-small cell lung cancer xenografts caused tumor shrinkage (60), demonstrating two methods of replacing this downregulated miRNA. AAVs may carry the risk of undesired immune responses and to date, no such therapeutic strategies have been employed in human cancer trials.

Nanoparticles are positively charged structures with diameters of 45-70nm that can be used to administer negatively charged miRNAs/mimics to target tissues. This structure confers greater miRNA stability, allows their slow release for prolonged miRNA targeting and avoids the possible immunogenicity associated with AAVs. These characteristics suggested their potential to be administered intravenously to humans. Davis et al. (61) recently published the preliminary results of the first human clinical trial using this delivery system. This Phase I study of patients with advanced solid tumors used the iv administration of an RNAi encapsulated in nanoparticles that targets the miRNA of ribonucleotide reductase (RRM2), a protein overexpressed in many solid tumors. The nanoparticles also contained surface transferrin protein targeting ligands (present on tumor cell surfaces) allowing specific delivery to tumors. The study demonstrated effective uptake of the RNAi to target tissue and efficient knockdown of its target gene. Response data is not yet mature.

5. PERSPECTIVE

The clinical relevance of miRNAs is clearly reflected by the fact that in the ten years since initially being linked to malignancy, they have progressed from discovery to biomarker and drug development programs and remain at the forefront of research into tumour biology.

Their role as biomarkers is promising particularly with regards to circulating miRNAs, which offer a potentially less invasive method of diagnosing cancer, assessing risk of relapse, and predicting and potentially following response to therapy. Guidance regarding the design of biomarker studies is rightly becoming more stringent (62) and additional appropriately planned studies involving larger sample sizes will be vital before specific miRNAs can be utilised clinically in this manner (63).

miRNA-modulating agents represent a new class of therapeutics, encompassing a wide range of mechanistic approaches including RNA interference and gene therapy, as well as complex delivery and tissue-targeting strategies. It is these last two points that have proved the biggest obstacle in the development of such agents. The human body’s natural barriers have hampered the systemic delivery of these drugs although this is being slowly overcome by innovative modifications to current agents and the design of new therapeutic structures. The generally widespread expression of many pertinent miRNAs has required the design of novel delivery systems to ensure tissue-specific targeting. Initial results from work by Davis et al. and developments in delivery systems by the pharmaceutical industry suggest that before long, the use of miRNA-based therapies may become common practise in the cancer clinic. Furthermore, linking evidence from prognostic and predictive biomarker studies with clinical trials involving miRNA-modulating therapies could lead to combined treatment strategies involving the use of such therapies with current treatments to maximise response and improve outcome.
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