miR-21 as a key regulator of oncogenic processes

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Abstract

Small non-coding miRNAs (microRNAs) are emerging as key factors involved in cancer at all stages ranging from initiation to metastasis. *MIRN21* is an miRNA gene that codes for the *miR-21* miRNA which has been found to be overexpressed in many tumour samples where it has been analysed. Whereas consistent overexpression of *miR-21* in tumours could be suggestive of functional effects of *miR-21* in cancer, more in-depth functional studies with *miR-21* are demonstrating that *mir-21* displays oncogenic activity and can be classed as an oncomir. Extensive efforts are underway to identify the downstream genes and gene networks regulated by *miR-21* and to identify the upstream factors that are regulating expression of *miR-21*. Even though *miR-21* is one of the most intensively studied miRNAs, for all miRNAs, our understanding of miRNA signalling pathways is currently in its early stages. The unravelling of such RNA signalling pathways and networks will be key to understanding the role that dysregulated miRNA functioning can play in oncogenic processes.

Introduction

miRNAs (microRNAs) and cancer

miRNAs are an abundant class of non-coding RNAs (~20–23 nucleotides) which can be expressed in a cell- and tissue-specific manner [1]. Since their discovery, research activity on this class of non-coding RNAs has dramatically accelerated due to their involvement in regulation of a wide range of cell and developmental processes [2]. Strong evidence has also emerged that miRNAs are key molecules involved in cancer initiation and progression [3,4]. As of September 2008, 695 miRNA sequences are listed for the human genome (miRBase: Database Release 12.0) [5].

miRNAs play an important role in gene regulation whereby they can target genes containing sequences of complementarity to the mature miRNA sequence and thereby regulate the level of expression of the targeted genes by a variety of mechanisms [6]. Disruptions of miRNA–target gene regulation, including genomic instability and impaired miRNA processing, have been associated with a growing range of cancers [7,8]. More than 50% of human miRNA genes are located in fragile sites and in the regions that are often associated with cancer, which provided an early indication of the potential importance of miRNAs in cancer [9,10].

miRNAs are often dysregulated in cancer cells and tissues with miRNA expression patterns correlated with tumour types and stages. In some cases, direct (functional) involvement of some miRNAs in cancer initiation and progression has been demonstrated [11,12]. While dysregulation of a miRNA–target gene relationship will have obvious implications for cancer if the target gene is an oncogene or tumour suppressor, it is also possible for miRNA dysregulation to cause indirect (secondary) effects on the expression levels of key genes or pathways implicated in cancer.

miRNA-21 gene and transcript (*MIRN21*, *miR-21*)

miR-21 was one of the first miRNAs detected in the human genome [13] and is known by a number of synonyms (*hsa-mir-21*, *MIR21*, *miR-21*, *MIRN21*, *miRNA21*). *miR-21* displays a strong evolutionary conservation across a wide range of vertebrate species in mammalian, avian and fish clades. In *Homo sapiens*, the *MIRN21* gene (DNA of pre-miR-21) is located on chromosome 17 (55273409–55273480, + strand) (Figure 1a) residing within the tenth intron of the gene *TMEM49* (transmembrane protein-49, also known as vacuole membrane protein-1) [14]. It has been demonstrated that a primary transcript containing *miR-21* (i.e. *pri-miR-21*) is independently transcribed from a conserved promoter that is located within the intron of the overlapping protein coding gene *TMEM49* [15].

Examination of several human miRNAs found that miRNAs are derived from capped and polyadenylated primary precursors (pri-miRNA). In the case of *miR-21*, a 3433 nt full-length *pri-miR-21* from which mature *miR-21* is derived. The *pri-miR-21* transcript contained a consensus AAUAAA polyadenylation signal between nucleotides +3394 and +3399 [13]. The stem–loop precursor of *miR-21* (*pre-miR-21*) resides between nucleotides +2445 and +2516 on the 3433 nt full-length *pri-miR-21* (Figure 1b).

A computational method to search for miPPRs (miRNA putative promoter regions) predicted such miPPRs for *miR-21* (*miPPR-21*) [15] in the 900 bp upstream of the TSS

Key words: cancer, gene regulation, microRNA (miRNA), *miR-21*, oncomir.

Abbreviations used: AP-1, activator protein 1; IL, interleukin; miPPR, microRNA putative promoter region; miRNA, microRNA; MMP, matrix metalloproteinase; PDCD4, programmed cell death 4; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA transcript; PTEN, phosphatase and tensin homologue deleted on chromosome 10; STAT3, signal transducer and activator of transcription 3; TGFβ, transforming growth factor β; TMEM49, transmembrane protein-49; TSS, transcription start site.

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Figure 1 | ***miR-21* as a key regulator of anglogenic processes**

(**a**) Location of *MIRN21* gene which codes for a 72-nt-long precursor *miR-21* (*pre-miR-21*) on chromosome 17q23.1. Chromosome ideogram produced using Bioconductor (GenomeGraphs package) [68]. (**b**) *pre-miR-21* sequence and stem–loop structure, mature *miR-21* is shown in bold. *pre-miR-21* sequence folded using Mfold [69]. (**c**) Mature *miR-21* sequences (predominant and minor forms) [5].

(transcription start site) reported previously [13]. Using primer extension approaches in HeLa and HL-60 mammalian cell lines, it was demonstrated that *miR-21* transcription was initiated 30 bp downstream of the conserved TATA box in *miPPR-21*, after PMA treatment (which induces *miR-21* expression in the HL-60 line) [15]. However, the previously described TSS for *miR-21* [13] was not detectable before or after the PMA treatment [15].

The primary *miR-21* transcript (*pri-miR-21*) transcribed by Pol II (DNA polymerase II) is processed by the nuclear RNAse III enzyme, Drosha and DGCR8 (DiGeorge syndrome critical region gene 8, a double-stranded RNA-binding protein) in the nucleus [16] forming the 72-nt-long stem–loop precursor (*pre-miR-21*) (Figure 1b), which is located to cytoplasm by the enzyme Exportin5 [17]. Following transport to cytoplasm, another RNase III enzyme, Dicer, recognizes this stem–loop precursor and cleaves it to yield a 22 nt miRNA–miRNA duplex. One of the strands (minor *hsa-miR-21**) is probably degraded, and the single-stranded mature miRNA destined for regulatory activity (predominant *hsa-miR-21*) (Figure 1c) is associated into a RISC (RNA-induced silencing complex)-like protein complex miRNP (miRNA ribonucleoprotein) [18] and is guided to any target mRNAs [mainly 3′-UTRs (untranslated regions)] containing near-perfect/imperfect complimentarily [19].

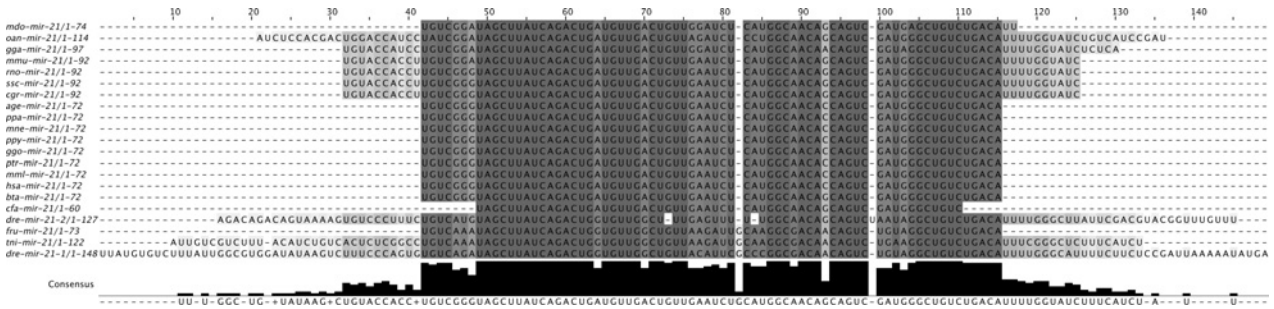
Although some miRNAs (e.g. *let-7*) have been found to be deeply conserved across species, there are also miRNAs that are species- or clade-specific. In the case of *miR-21*, multiple sequence alignments of the precursor and mature *miR-21* regions shows that both sequences are highly conserved across many species, possibly suggesting a deeply conserved role for *miR-21* in gene regulation (Figure 2).

Overexpression of *miR-21* is associated with many forms of cancers

The discovery of multiple miRNAs and the advent of high-throughput transcriptome profiling approaches for

Figure 2 | Evolutionary conservation of the *miR-21* precursor region (*pre-miR-21*) across 21 vertebrate species, spanning mammalian, avian and fish species

The RNA sequence of each *miR-21* precursor in each species is aligned with all of the other *miR-21* homologues in a T-coffee multiple sequence alignment [70,71]. Residues are shaded according to similarity, with darker shading indicating higher consensus. Species abbreviations: age, *Ateles geoffroyi*; bta, *Bos taurus*; cfa, *Canis familaris*; cgr, *Cricetulus griseus*; dre, *Danio rerio*; fru, *Fugu rubripes*; gga, *Gallus gallus*; mdo, *Monodelphis domestica*; mml, *Macaca nemestrina*; mmu, *Mus musculus*; oan, *Ornithorhynchus anatinus*; ppa, *Pan paniscus*; rno, *Rattus norvegicus*; ssc, *Sus scrofa*; tni, *Tetraodon nigroviridis*.



miRNAs have facilitated comparative analysis of miRNA expression profiles in tumours and cell lines associated with cancer, with those of normal cells/tissues. Strikingly, *miR-21* has been found to be overexpressed in the vast majority of cancer types analysed, although one study has found that inhibition of *miR-21* expression was associated with increased cell growth in cancer cells [20].

Transcript profiling studies of miRNA expression levels across tumour samples or cancer cell lines have revealed that *miR-21* is up-regulated in cell lines/tissues specific to glioblastoma [21], lung cancer [22,23], stomach cancer [22], oesophageal cancer [24], prostate cancer [22], colon cancer [22,25], ovarian carcinoma [26], cholangiocarcinoma [27], B-cell lymphoma [28], hepatocellular carcinomas [29,30], cervical cancer [31], uterine leiomyomas [32], head and neck cancer [33], chronic lymphocytic leukaemia [34], pancreas cancer [22,35], squamous cell carcinoma of the tongue [36], papillary thyroid carcinoma [37] and breast cancer [38,39]. In addition, another study showed that overexpression of *miR-21* in primary breast cancer samples is associated with advanced clinical stage, lymph node metastasis and poor prognosis [40]. However, although such studies clearly demonstrate that *miR-21* is deregulated in samples where cancer is at an advanced stage, such studies do not prove any causal role for *miR-21* in cancer aetiology. From a functional perspective, the entire field of cancer research is challenged by the nature of cancer itself because of the difficulty of identifying and profiling samples which are at the earliest stages of cancer initiation and development (i.e. single-cell or few-cell stages) [41]. Nonetheless, such studies to identify miRNA profiles associated with specific cancer types are very useful for classifying cancers based on miRNA profiles and for identification of cancer-specific 'biomarkers' that can be used in cancer diagnosis and treatment assessment.

Functional demonstrations that *miR-21* is an oncomir

Although there has been extensive miRNA profiling of cancer compared with normal tissues and cells, which have identified *miR-21* as a key molecule (or biomarker) associated with a wide range of cancers, there have been fewer functional studies that demonstrate cause–effect relationships between *miR-21* and neoplastic transformation. The following provide an overview of functional studies to date (e.g. assaying function after *miR-21* knockdown by antisense inhibitors) conducted with *miR-21* that strongly suggests that *miR-21* has oncogenic activity.

(i) Knockdown of *miR-21* in cultured glioblastoma cells triggers activation of caspases and leads to increased apoptotic cell death, suggesting that expression of *miR-21* can act as an anti-apoptotic factor [21].

(ii) Knockdown of *miR-21* in breast cancer MCF7 cells led to suppression of cell growth *in vitro* and tumour growth in the xenograft mouse model. Cell growth suppression was accompanied by increased apoptosis and decreased cell proliferation (possibly due to down-regulation of Bcl-2 protein expression) [42].

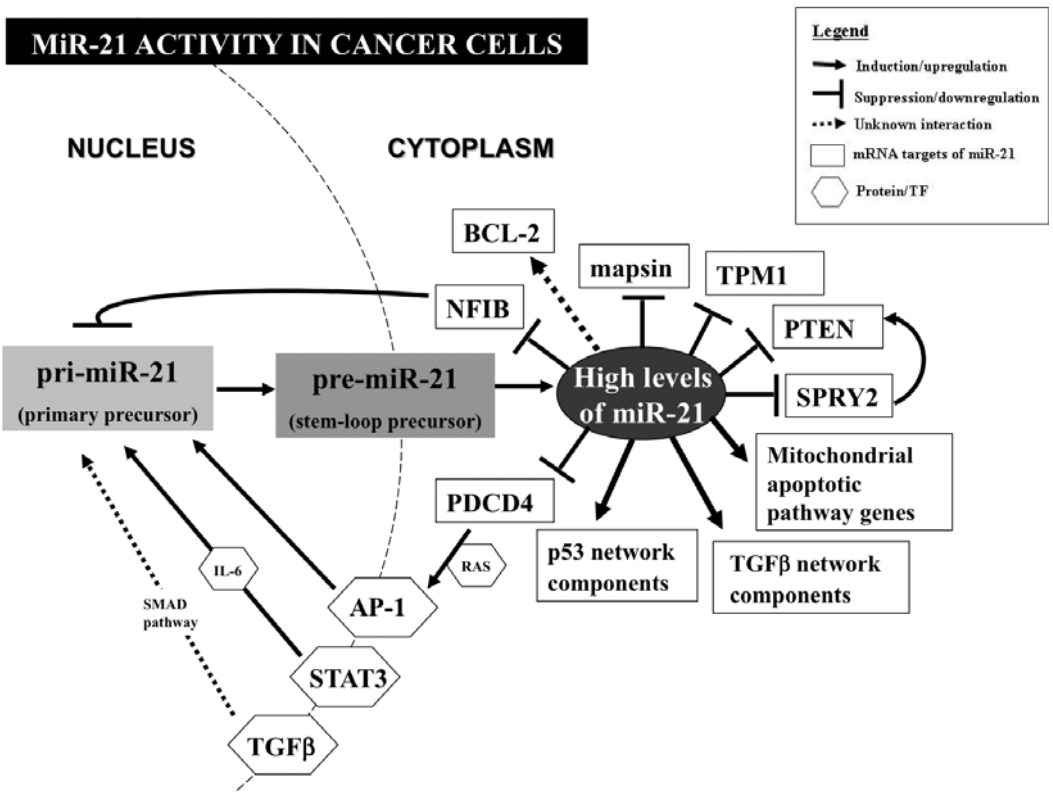
(iii) Knockdown of *miR-21* in metastatic breast cancer MDA-MB-231 cells significantly reduced invasion and lung metastasis [11].

(iv) Knockdown of *miR-21* in hepatocellular cells increased expression of the PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor (a direct target of *miR-21*) (Figure 3), and decreased tumour cell proliferation, migration and invasion. An increase of *miR-21* expression in hepatocellular cells led to increased tumour cell proliferation, migration, and invasion [30].

(v) In colorectal cell lines, an inverse correlation of *miR-21* and the tumour suppressor PDCD4 (programmed cell

Figure 3 | ***miR-21* activity in cancer cells**

Schematic representation of validated targets and interactions of *miR-21* in cancer cells based on current literature. NFIB, nuclear factor I/B; TF, transcription factor.



death 4) protein (translational repression of *PDCD4* by *miR-21*) has been observed [43]. Knockdown of *miR-21* leads to an increase in PDCD4 protein levels and reduced invasiveness, while *miR-21* overexpression led to increased invasiveness and metastasis [43].

(vi) Increasing *miR-21* expression in myeloma cells [in the absence of IL (interleukin)-6] significantly reduced their apoptosis levels [44].

(vii) In a screen of miRNAs in HeLa cells treated with a library of miRNA inhibitors, it has been found that, along with other miRNAs, *miR-21* inhibition caused a decrease in cell growth [20].

(viii) Antisense inhibition of *miR-21* caused significant apoptotic cell death in neuroepithelial cells through significant activation of caspases detected by pan-caspase assay. *miR-21*-antisense-treated cultures also showed high LDH (lactate dehydrogenase) release as an indication of apoptotic cell death. *miR-335*-antisense-treated neuroepithelial cells showed resistance to apoptosis after ethanol treatment and prevented cell death caused by *miR-21* suppression, indicating an antagonistic effect of *miR-335* to *miR-21* [45].

(ix) Using RT (reverse transcripion)–PCR to detect expression of mature *miR-21*, *miR-21* expression was detected in 344 fresh tumour samples collected from patients diagnosed with primary breast cancer. Considering features such as disease stage, tumour grade, histology, hormone receptor status and lymph node involvement, high *miR-21* expression has been found to be linked to aggressiveness of the disease, high tumour grade, negative hormone receptor status, poor disease-free survival in early stage patients and ductal carcinoma, but no clinical value for prognosis [14].

Overall, these studies indicate that knockdowns of the expression of *miR-21* can lead to functional effects clearly linked with cancer initiation and progression. Such studies suggest that *miR-21* has some oncogenic activity, which, if removed, inhibits the development of cancer-associated phenotypes in cell lines.

What downstream pathways and genes are regulated by *miR-21*?

Although it is known that *miR-21* is overexpressed in cancer cells/tissues and has oncogenic activity in terms of neoplastic transformation, little is known regarding the genes and pathways downstream that are regulated by *miR-21*, particularly those which, if misregulated, can trigger neoplastic cellular growth.

Much of the early efforts to identify downstream targets of miRNAs have been based on computational target prediction algorithms, and the number of predicted miRNA target genes vastly outnumbers the number of miRNA target genes

that have been actually validated in wet-lab experiments [46]. This is evident from the fact that there are over 600 known miRNAs potentially (i.e. based on computational screens) targeting up to 30% (more than 5300 genes) of protein-coding genes in the human genome [47], yet there are only 461 experimentally validated miRNA–target gene demonstrations in TarBase [48].

A number of tumour-suppressor genes have been found to be targeted by *miR-21*, supporting its proposed oncogenic role in cancer (Figure 3). A study using two-dimensional proteomics, luciferase reporter analysis and Western blot assays to screen for translational suppression of target genes due to *miR-21* has validated the tumour suppressor gene *TPM1* (tropomyosin-1) in breast cancer as a target of *miR-21* [49]. Target validation studies in breast cancer samples (cell lines and tumours) and colorectal cancer cells on putative *miR-21* targets have demonstrated a link between *miR-21* expression levels and the p53 tumour suppressor, and also demonstrated that the tumour suppressors *PDCD4* and *maspin* are targets of *miR-21* [11,43,50]. Other candidate targets of *miR-21* that have been recently validated include the *PTEN* tumour suppressor in hepatocellular carcinoma cells [30] and *SPRY2* [51] which is also known to induce up-regulation of *PTEN* [52]. In addition, an indirect regulation of *Bcl-2* by *miR-21* has also been shown in breast cancer [53]. A growing body of *miR-21* targets are now being validated, including recent studies in glioblastoma cells and cervical cancer cell lines, which identified a range of *miR-21*-targeted genes [including the *HNRPK* (heterogeneous nuclear ribonucleoprotein K) and *TAp63* genes which are components of the p53, TGFβ (transforming growth factor β) and mitochondrial apoptosis pathways] [54]. Recently, it has been shown that in glioma cells *miR-21* negatively regulates MMP (matrix metalloproteinase) inhibitors, which causes activation of MMPs, causing invasiveness of cancer cells [55].

Microarray studies investigating effects of knockdown of *miR-21* in the breast cancer cell line MCF7 have identified a range of 737 genes whose mRNA levels are affected (402 genes up-regulated, 335 genes down-regulated) by loss of *miR-21* [50]. The advent of SILAC (stable isotope labelling by amino acids in cell culture) approaches for the identification of targets of miRNAs indicates that there can be both a knockdown in the levels of the mRNA transcript and the protein [55–57]. This indicates that microarray-based approaches in combination with techniques to assay protein levels will be of use for identification of the downstream genes and gene networks regulated by miRNAs in different cell line backgrounds.

What upstream pathways and genes regulate *miR-21*?

The functional identification of regulatory genes upstream of miRNAs which are responsible for controlling the spatial and temporal expression of specific miRNAs is also in its early stages. In this regard, the possible involvement of the *STAT3* (signal transducer and activator of transcription 3) gene in regulation of miRNA genes was investigated using computational analysis of evolutionary conserved putative STAT3-binding sites in regulatory regions of miRNA genes. STAT3 is a transcription factor that mediates IL-6 signalling and has been shown to be involved in cellular transformation and oncogenesis. Among the miRNAs analysed, the *miR-21* gene region contained two consensus STAT3-binding sites ~800 bp upstream of its TSS, both of which are highly conserved in vertebrates [44]. They have shown that *miR-21* expression is controlled by an upstream enhancer element containing these two evolutionarily conserved STAT3-binding sites. Induction of *miR-21* expression by IL-6 requires STAT3, implicating *miR-21* in the oncogenic potential of STAT3 [44]. Chromatin immunoprecipitation experiments in human myeloma cells (XG-1) have demonstrated that STAT3 is recruited to the *miR-21* regulatory region in response to IL-6. This was validated by inhibition of *miR-21* promoter activity (decreased primary *miR-21* transcript levels) after STAT3 knockdown. The experiments demonstrated that *miR-21* gene transcription is controlled by IL-6, which requires STAT3 regulation of *miR-21* upstream enhancer [44]. These recent findings regarding regulation of *miR-21* may indicate co-operation of STAT3 and *miR-21* in tumorigenesis.

The transcription factor AP-1 (activator protein 1) is known to be an important regulator of cell proliferation, apoptosis and invasion [58,59] and has also been shown to activate *miR-21* transcription [15]. Bioinformatic approaches have revealed highly conserved potential AP-1-binding sites in *pri-miR-21*, suggesting that *miR-21* is a possible transcriptional target of AP-1. AP-1-induced expression of *miR-21* causes down-regulation of *miR-21* target, *PDCD4*, which is required for stimulation of AP-1 in response to the Ras oncoprotein. This indicates an autoregulatory loop between *PDCD4* and *miR-21* for controlling AP-1 activity in Ras-transformed cells. One of the AP-1-binding sites has been validated by functional assays as the major Ras-responsive element of the *miR-21* gene promoter. AP-1-induced *miR-21* expression causes up-regulation of rat thyroid cell growth and down-regulation of *miR-21* targets: tumour-suppressor genes *PDCD4* and *PTEN* [15,60].

In a separate analysis of endogenous *miR-21* expression, TGFβ1 has been shown to up-regulate *miR-21* expression. Positive correlation of high *miR-21* expression with TGF-β1 in tumour samples has been found which may indicate regulation of *miR-21* expression by TGF-β1 [14]. This finding is in concordance with a recent report indicating up-regulation of *miR-21* expression by TGF-β1 through the Smad signal transduction pathway [61].

miR-21 as a cancer biomarker

A wide range of studies have now shown that miRNAs can act as more powerful cancer biomarkers (e.g. for cancer type, prognosis, responsiveness to therapy) than mRNAs or proteins [22,27,32,38]. For instance, overexpression of *miR-21* in pancreatic endocrine and acinar tumours is strongly associated with both a high Ki67 cell proliferation index, and presence of

liver metastasis [62]. Previously, it has been demonstrated that high *miR-21* expression is associated with poor survival and poor therapeutic outcome of colon adenocarcinomas [63].

Analysis of the effects of anticancer chemotherapeutic agents (e.g. 5-fluorouracil, gemcitabine) in relation to miRNAs has demonstrated that inhibition of *miR-21* increased sensitivity to gemcitabine-induced apoptosis [27], whereas *miR-21* expression is increased in response to cellular treatment with 5-fluorouracil [64,65]. Pioneering studies are now underway whereby combinatorial approaches to cancer treatment based on modulation of miRNAs (such as *miR-21*) in conjunction with conventional therapeutics [e.g. the cytotoxic agent S-TRAIL (soluble tumour-necrosis-factor-related apoptosis-inducing ligand), or libraries of anticancer compounds] are showing promise [66,67].

miR-21 is also detected in biological fluids such as in serum. High *miR-21* expression was found in serum samples of DLBCL (diffuse large B-cell lymphoma) patients compared with healthy control samples. Also, *miR-21* expression in disease samples was associated with relapse-free survival, but not overall survival. These approaches are highlighting the potential use of miRNAs as non-invasive diagnostic markers in cancer [28].

Conclusions

The discovery of the important role of miRNAs in cancer has opened up a new era of cancer investigations that take into account new and emerging knowledge regarding the RNA signalling systems within eukaryotic cells such as mammalian cells. Among the many miRNAs already identified as regulators of neoplastic transformation, invasion and metastasis, *miR-21* has emerged as key miRNA (oncomir) which is dysregulated in many cancers. Although there are many predicted targets of miRNAs available using online tools, only a handful are validated for miRNAs such as *miR-21* (Figure 3).

Even less is known regarding the regulatory networks in which miRNAs (operating in union or in concert) are embedded. Moreover, tissue-specific expression of miRNAs and combinatorial effects of different miRNAs on a particular target gene have to be taken into account for a more comprehensive understanding of miRNA functioning. Hence, understanding in healthy cells, tissues and individuals how *miR-21* is regulated and how *miR-21* regulates downstream target genes will be a prerequisite for rational design of cancer therapeutic strategies based on modulation of *miR-21* expression.

Nonetheless, even with available knowledge and resources, it is clear that studies on miRNA expression profiling and targets in cancer samples are proving worthwhile for developing effective cancer biomarkers for diagnosis and individualized medicine (e.g. how biomarkers can give an indication for likely response of a cancer patient or cohort to a specific drug or treatment). Depending on the cancer and its stage of development, therapeutics can target specific stages and types of tumours. The involvement of miRNAs in all stages of cancer development from initiation to progression and metastasis opens the possibility for miRNA-targeted or miRNA-based therapeutics. Examples of such therapeutics include silencing oncomirs or gene therapy approaches based on re-expression of miRNAs that are down-regulated in cancer cells.

miRNAs such as *miR-21* have heralded a new era of research on 'RNA signalling pathways' which now have to be elucidated and related to what we know regarding more conventional protein signalling pathways (Figure 3). The integration of such research into a systems-based understanding of gene networks that underlie normal and neoplastic cell division should allow for the identification of new approaches for cancer diagnosis and therapy.

Funding

Supported by Cancer Research Ireland and the Irish Research Council for Science, Engineering and Technology (IRCSET).

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Received 27 January 2009
doi:10.1042/BST0370918