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Abstract

Whilst the promise of RNA interference (RNAi) continues to expand in the therapeutic environment, the utility of the endogenous RNAi mechanism, microRNAs (miRNA), in diagnostics has been proven with the successful commercialisation of a biopsy assay for the identification of cancer of unknown primary origin (CUP). The implications and consequences in personalised medicine arising from this highly sensitive and specific approach are substantial, however the true potential is only beginning to be understood, as reports of disease-specific miRNA signatures in circulating lymphocytes, blood plasma/serum, lung sputum, saliva and urine have been described, even in diseases where no diagnostic tools exist. This review summarises the various approaches in miRNA biomarker discovery and summarises key findings in the field with the highest potential for clinical development.

Introduction

MicroRNA's (miRNA) are a novel class of endogenous RNA regulators of gene expression. The 721 miRNA so far described in man (miRBase v.14, http://www.mirbase.org/) are found in non-coding genes individually or in clusters, with some exceptions processed from protein-coding transcripts. A transcript encoding a miRNA (primary miRNA) is processed in the nucleus into the miRNA precursor (pre-miR), a stem-loop hairpin RNA of ~70 nucleotides.

The pre-miR is actively exported to and processed into the mature miRNA in the cytoplasm. This double stranded RNA (dsRNA) features i) a length usually between 21-25 bp, ii) at least one mismatch and iii) at least one 3' end overhang. One strand is then loaded onto an Argonaute protein, forming the miRNA-induced silencing complex (mi-RISC) [1].

In a mechanism almost identical to that of short interfering RNAs (siRNA), mi-RISC complexes 'scan' the 3' un-translated regions (UTR) of mRNA's for complementarity to the miRNA. Substantial complementarity identifies a miRNA recognition element (MRE) and miRISC sequesters the mRNA preventing translation, or mediating eventual degradation [2]. Bases 2-7 from the 5' end of the miRNA, known as the 'seed' region, drive most MRE identifications [3], however this is not exclusive [4]. The seed region size suggests that hundreds of proteins might be regulated by each miRNA and algorithms are routinely used for bioinformatic target prediction [5]. However, additional mechanisms of miRNA-mediated gene expression regulation exist, including mRNA 5'-UTR interactions and chromatin modification [6].

The plethora of miRNA targets suggests a tight network of molecular interactions orchestrating protein expression, with miRNAs constituting crucial nodes. This is manifest in the established roles of specific miRNAs in cellular and physiological processes in the central nervous system, immune system, heart and skeletal muscle, diabetes and metabolism, etc. [7]. Additionally, at least 176 miRNAs are also found in human viruses (miRBase v. 14), which also encode protein suppressors of RNAi [8, 9]. Similar functions have also been described for intracellular bacterial pathogens, so far only in plants [10]. Importantly, both miRNA and their mRNA targets are highly conserved between species. Thus, miRNA signatures associated with a specific biological condition (e.g. inflammation) tend to be conserved across species of interest. Notwithstanding the therapeutic opportunities presented in restoring deregulated miRNA expression, there is the potential for miRNA signatures to be

used not only as cross-species biomarkers of disease, for toxicology analysis and drug safety monitoring, but also as prognostic factors, to assist in the classification of disease and as markers of drug efficacy.

Approaches and considerations in miRNA profiling

Numerous specialist and commercially available methodologies for miRNA transcriptome (miRnome) profiling have been developed. These can be broadly grouped into three categories: quantitative polymerase chain reaction (qPCR), hybridization assays and sequencing (see table 1). The technological differences between each technique may result in profiling data that differ between platforms; e.g. an adenosine to inosine editing event [11] which could impact on a qPCR assay could go undetected through microarray profiling, as has been highlighted in mRNA profiling [12]. The most advanced and sensitive method currently available is next generation sequencing (NGS), a collection of platform technologies that perform up to 50 x 10^9 base reads per run [13], building a comprehensive, quantitative picture of microRNA and other small non-coding RNA profiles.

Table 1: Comparative assessment of microRNA profiling technology platforms

The nature of the sample can similarly impact on miRnome profiles. Thus, whereas in isolated lung epithelia nuclear factor kappa B (NF-κB) pathway-mediated inflammation results in induction of miR-146 [14], this is not detected at the whole lung level [15]. On the other hand miRNA levels have been shown to be diurnally expressed in the brain [16], the retina [17] and the liver [18], with changes detected either at the pre-miR or mature miRNA

level [19]. In the context of disease, in transient focal cerebral ischemia, the observed miRnome changes were reversed within 48 hours [20]. MicroRNA response onset might also precede cellular responses *in vivo* [15], whereas *in vitro* rapid oscillations (T < 1 hr) have been reported [21]. Collectively, these studies indicate that miRNA profiling should be approached with the same considerations pertinent to classical gene expression profiling.

miRNA profiling in disease; the cancer paradigm

By the end of 2009 over 1600 peer-reviewed articles and 500 reviews had been published associating miRNA deregulation in cancer, with four cancer biomarker assays having become commercially available. These covered the identification of pancreatic cancer over pancreatitis [22], the differentiation of squamous vs. non-squamous lung cancer [23], malignant pleural mesothelioma from cancers of the lung and the pleura [24], and the identification of the tissue of origin for metastatic cancers of unknown primary origin (CUP) [25]. With assay sensitivity and specificity in excess of 90%, these assays addressed up to 30% error rates in diagnosis, an important step forward towards personalised medicine. Crucially, several studies have now demonstrated associations of miRnome signatures with various aspects of cancer, including disease progression, survival prediction and treatment selection and efficacy (for an in depth review see [26]).

Disease diagnosis and classification through biopsy miRNA profiling

In a study examining the involvement of miRNA in primary biliary cirrhosis (PBC) a microarray platform was used to demonstrate the deregulation of 35 miRNA in six end-stage PBC patients vs. five healthy controls [27]. The findings for at least three downregulated

(miR-122a, miR-26a and miR-99a) and three upregulated (miR-299-5p, miR-328 and miR-371) miRNAs were then validated in an expanded sample population by qPCR. Whilst of the affected miRNAs, loss of miR-122 expression alone has been shown to be associated with liver disease (see table 2), the remaining PBC miRnome signature was not directly ascribed to the disease on account of the complex physiology of end-stage PBC.

The extent to which a biopsy can always yield a diagnostic miRnome signature was put to the question in a study investigating miRNA signatures in mild asthma [28]. qPCR screening in bronchial biopsies failed to identify any significant changes either in disease, or following treatment. The absence of disease or pharmacodynamic signatures was ascribed to the heterogeneity in the cell population constituting the biopsy and the potential lack of treatment effect on mild asthma miRNA homeostasis. Thus, whilst homogeneous tissue biopsies such as from the liver might yield clear disease signatures, this study demonstrates the urgent need for methodologies that allow cell-type specific analysis of biomarker expression.

Elsewhere, the expression of 365 miRNAs was evaluated by Taqman as a means of predicting renal allograft rejection and function [29]. Of the 17 miRNAs differentially expressed in a training sample set (4 normal, 3 acute rejection biopsies), 6 were validated using an independent set of 26 biopsies (9 acute rejection, 17 normal), identifying over-expression of miR-155 and miR-142-5p as acute rejection status predictors at 100% sensitivity and 95% specificity. The inflammatory cell component in this disease was considered accountable for the observed upregulation of miR-155, miR-142-5p and miR-223, well-described components of the lymphocyte compartment miRnome [30]. Curiously, the authors observed these miRNAs as highly expressed also in peripheral blood mononuclear cells (PBMC) [29], suggesting biomarker identification might be possible to carry out peripherally.

miRNA biomarkers in circulating cells and biofluids

To date there is no single laboratory-based assay for effective diagnosis of systemic lupus erythematosus (SLE). Moreover, discrimination of SLE from immune thrombocytopenic purpura (ITP) is complicated by common symptomatology and similar lack of diagnostic assays. Using microarrays, 16 miRNAs differentially regulated in SLE patient PBMCs was observed [31]. Moreover, the reduction of expression for 8 miRNA was found to correlate with increase in SLE severity. Similarly, 19 miRNA were found to be deregulated in ITP. Whilst a common signature was observed for 11 miRNA, downregulation of miR-184 and upregulation of miR-198 and miR-21 were specific to SLE, whereas downregulation of 6 miRNAs was specific to ITP. Whilst a large training set was used, biomarker validation was not carried out. Moreover, comparison to the PBMC miRnome profiles of other autoimmune diseases is needed to substantiate the utility of these signatures in SLE and ITP diagnosis and discrimination. Encouragingly, deregulation in the expression of 66 miRNA in renal biopsies of Lupus nephritis patients, a common complication of SLE [32], has also been described, though the PBMC signature was not reflected in the kidney.

Table 2: miRNA biomarker assays proposed for physiological condition and disease diagnosis.

MicroRNA profiling studies have also been extensively carried out in rheumatoid arthritis (RA). Similar to acute allograft rejection, miR-155 and miR-146a was upregulated in RA synovial fibroblasts, synovial tissue and synovial macrophages when compared to

osteoarthritis (OA) samples [33]. Elsewhere, miR-146a was also observed to be overexpressed in RA synovial tissue as compared to OA, primarily but not exclusively in the lymphocyte infiltrate [34]. In addition to miR-155 and miR-146a, increased miR-132 and miR-16 expression was also observed in a separate study in RA patient PBMC's [35], whereas in peripheral CD4+ T lymphocytes miR-223 was the only deregulated miRNA [36]. miR-146a is emerging as a hallmark of severe inflammation [14] whose deregulation might underlie aspects of aberrant proinflammatory cytokine expression [35, 37]. However, it is worth noting that whilst the same cellular infiltrate might mediate similar tissue damage, the precise immunological milieu may differ sufficiently to warrant distinct miRnome profiles [38].

An inflammatory and innate immune response component is also found in Alzheimer's disease (AD). Several miRNAs previously linked to neurogenesis and neurodifferentiation were affected in a spatiotemporal manner in diseased patient brain samples and/or over different disease stages [39]. However, these changes were not reflected in cerebrospinal fluid (CSF) samples, but rather underexpression of miR-146b, a miRNA associated with inflammation, as well as miR-181a and miR-142, two miRNA previously associated with T cell activation and differentiation, was documented. The authors proposed that these changes were on account of upregulation of innate immune pathways in resident CSF lymphocytes as opposed to cellular infiltrate [39].

On account of reports of <70 nucleotide RNA in circulating blood, Mitchell *et al.* cloned and sequenced such molecules from the plasma of a healthy donor [40]. Amazingly, 93% of these clones corresponded to known miRNA at concentrations of 0.9-1.3 e5 copies per ul of plasma, and were protected from RNase activity. Aiming to detect tumour-derived miRNA in plasma, a human prostate cancer cell line xenograft was implanted in immunocompromised mice. The process had no impact upon canonical plasma miRNA levels in the mouse, but two

human-specific miRNA (miR-629* and miR-660) overexpressed in the xenograft were detected. By assaying for six biopsy-overexpressed miRNAs not present in healthy patient plasma the authors identified that miR-141 could distinguish diseased from healthy individuals with 60% sensitivity and 100% specificity. By re-examining previously published data on the miRnomes of various human cancers [41], retracing the evolutionary conservation of miR-141 back to zebrafish, and performing sequencing analyses of prostate stromal vs epithelial cell miRnomes, the authors demonstrated that this miRNA is representative of epithelial tissue [40]. Given that other epithelial cancers also overexpress miR-141 [41], it remains to be seen if plasma detection of miR-141 also is diagnostic of prostate cancer.

At the same time, Chen *et al.* were employing NGS to investigate the broad utility of miRNA as serum biomarkers [42], documenting species- and sex-specific miRNA profiles in serum, and signatures of other cancers and diseases such as type II diabetes. Importantly, 4% of the detected miRNA were unique to blood cells whereas 6% were unique to serum, suggesting serum miRNAs are not cellular debris artefacts. In addition, 55 of the miRNA overexpressed in lung cancer patient sera were also detected in colorectal cancer patient sera. Unexpectedly, more than a third (38.5%) of the lung cancer-overexpressed miRNA were also detected in type II diabetes samples, probably a consequence of the common underlying inflammatory component, as alluded by the overexpression of inflammation-associated miRNA (e.g. miR-146a) in both sample sets. Collectively, these data support the tenet that serum-based disease detection by means of miRNA profiling will require multiple as opposed to single or paired target expression assessment.

Following these reports, serum or plasma miRNA detection has been proposed for ovarian cancer [43] and pregnancy [44]. However, circulating miRNA levels might also be highly sensitive to tissue damage. Following induction of hepatotoxicity in BALB/c mice through paracetamol overdose miRNA deregulation was observed in plasma as well as in the liver.

Curiously, miR-122 was substantially downregulated in the liver but upregulated in plasma in a time- and dose-dependent fashion. This profile manifested in advance, and with higher sensitivity to plasma alanine aminotransferase (ALT) changes, a classical biomarker of liver damage [45]. Indeed, evidence from multiple angles now suggests that liver underexpression of miR-122 appears to be a hallmark of liver disease [46-49].

Exosomal and mucosal miRNA

What is the origin of these miRNAs in circulation? The relative stability of serum miRNAs [39, 41] has given rise to hypotheses of disease-communicating complexes [50]. Such functions are believed to be mediated by cell-derived microparticles such as exosomes. These are membranous vesicles of 40-100 nm diameter containing proteins, mRNA and miRNAs [51], believed to extensively mediate horizontal communication between numerous cell types both *in vitro* and *in vivo*, including stem cells, tumour cells, B cells, dendritic cells, mast cells, platelets, placental trophoblasts, glial cells and neurones [51-58]. In ovarian cancer the density of circulating exosomes has been reported to increase with disease state, whilst tumour-specific miRNA have been identified in these structures [54]. Similar data have been reported in lung adenocarcinoma, though in this case no correlation could be drawn against disease state [55]. Such exosome-associated miRNA signatures have been proposed as diagnostic biomarkers for both diseases [55, 59].

Disease-associated signatures have also been recovered from mucosal biofluids, raising the tantalising prospect of non-invasive biomarker applications for miRNAs. Thus, miR-21 expression analysis in non-small cell lung cancer (NSCLC) patient sputum could detect the disease with ~70% sensitivity and 100% specificity [60]. As in serum studies, sputum miRNAs were also resistant to RNase degradation. Similarly, miRNA-126 and miR-182 were

found to be overexpressed in bladder cancer urine samples [61], with the miR-126:miR-152 ratio being 72% sensitive and 82% specific to bladder cancer. Elsewhere, saliva samples were shown to be sources of relatively RNase-resistant miRNA, with levels of miR-125a and miR-200a able to differentiate oral squamous cell carcinoma patients from healthy controls [62]. As with circulatory miRNA, early evidence suggest that the source of salivary miRNA may indeed be exosomes [63].

Conclusions

Whilst commercialisation of miRNA-based assays for cancer has provided proof-of-concept for the utility of these biomolecules in diagnostics [22-25], the full potential behind this approach is only now beginning to emerge. The unexpected presence of miRNA in circulation [40, 42-45] and mucosal secretions [60-62] suggests that a wide variety of diseases might be possible to assay for using miRNAs. In addition, the kinetics of circulatory miR-122 levels in response to liver damage [45] indicate that miRNA might present novel, significantly improved, cross-species biomarker opportunities in toxicology and drug safety assessment. Human or *in vivo* data supportive of the utility of miRNA monitoring on drug efficacy might be presently lacking [15, 28, 36], however, the studies conducted so far have served to highlight a need for improved analytical procedures with regards to tissue sample collection. As a wide variety of diseases involve complex cellular environments, ‘whole biopsy’ analysis serves to identify composite signatures that may or may not reflect a range of diseases with commonly engaged processes, e.g. inflammation, complicating diagnosis. It is encouraging, however, that irrespective of these challenges, miRNA profiling might indeed be the road to end the quest for precise diagnostics for previously unaddressed diseases and personalised medicine.

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Table 2: miRNA biomarker assays proposed for physiological conditio

Condition
Pancreatic ductal adenocarcinoma vs pancreatitis †
Squamous vs. non-squamous lung cancer †
Malignant pleural mesothelioma vs. pleural and lung cancers †
Identification of cancer of unknown primary origin †
Primary biliary cirrhosis
Renal allograft rejection
Rheumatoid arthritis vs. osteoarthritis
Rheumatoid arthritis
Rheumatoid arthritis
Alzheimer's disease
Prostate cancer
Non-small cell lung carcinoma
Ovarian cancer
Pregnancy
Liver damage ‡
Ovarian cancer
Lung adenocarcinoma
Non-small cell lung carcinoma
Bladder cancer
Oral squamous cell carcinoma

†: Commericalised

‡: Mouse study on paracetamol toxicity

ı and disease diagnosis.

Tissue sampling	Assay target(s)	Sensitivity / Specificity (%)	Reference
Pancreas biopsy	miR-217, miR-196a	95 / 95	[22]
Lung biopsy	miR-205, miR-21	91 / 97	[23]
Tumour biopsy	miR-200c, miR-193a-3p, mi	95 / 96	[24]
Tumour biopsy	48 miRNA panel, see refere	90 / 99	[25]
Liver biopsy	miR-122a, miR-26a, miR-99	Not available	[27]
Kidney biopsy	miR-155, miR-142-5p	100 / 95	[29]
Synovial tissue	miR-155, miR-146a	Not available	[33, 34]
PBMC's	miR-16, miR-132, miR-146a,	Not available	[35]
Peripheral CD4+ T lyr	miR-223	Not available	[36]
CSF	miR-146b, miR-181a, miR-1	Not available	[39]
Blood plasma	miR-141	60 / 100	[40]
Blood serum	miR-25, miR-223	Not available	[42]
Blood serum	miR-21, miR-29a, miR-92, m	Not available	[43]
Blood plasma	miR-526a, miR-527, miR-52	Not available	[44]
Blood plasma	miR-122, miR-192	Not available	[45]
Blood serum exosom	miR-21, miR-141, miR-200a,	Not available	[54]
Blood plasma exoson	miR-17-3p, miR-21, miR-10	Not available	[55]
Lung sputum	miR-21	70 / 100	[60]
Urine	miR-126:miR-152 ratio	72 / 82	[61]
Saliva	miR-125a, miR-200a	Not available	[62]