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The Future of Biomarkers is Micro: the Emerging Role of Micro-RNAs in Metabolic Diseases

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Genetics 101

Genes are a heredity unit coding for specific traits and characteristics passed down from parents to offspring. DNA stores this genetic information which first must be converted into a usable form; transcription is the process of creating RNA from DNA. The newly formed RNA is then used as a blue-print for protein synthesis during translation (Figure 1). Over the past couple of decades, an additional layer of complexity has been added to this central dogma of molecular biology. The once considered "junk" or non-coding regions of DNA has now been shown to code for micro-RNAs (miRNAs). miRNAs are evolutionally conserved, small RNA molecules that regulate gene expression and ultimately protein synthesis [1]. They control the amount of RNA transcript available for protein production, through complementary base pairing, miRNA bind to a specific RNA sequence which results in suppressed translation or direct targeting of the RNA for degradation [2] (Figure 1).

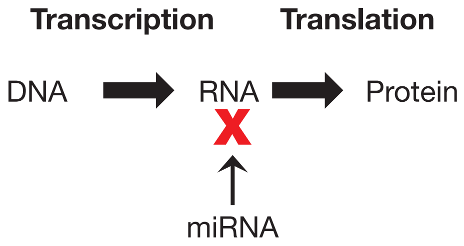

Figure 1. Genetic central dogma and the role of miRNAs as post-transcriptional negative regulators of gene expression.

Unique miRNAs have several target genes and can regulate the expression of an entire signaling pathway at multiple points. Therefore it is estimated that approximately 60% of all human genes are influenced by miRNAs [3]. miRNAs are typically 18-25 nucleotides in length, and can be released into the blood stream where they are detected in serum or plasma. Circulating miRNAs can be packaged into small compartments called microvesicle [4], associated with lipoproteins [5], or linked to an RNA-binding protein [6], rendering miRNA much more stable than other genetic material such as RNA. More importantly, many soluble miRNAs are derived from a specific cell or tissue, making miRNAs ideal biomarkers.

Application as a Biomarker

Since miRNAs are abundant in bodily fluids; blood and urine collection are practical, non-invasive manners for sampling. Circulating miRNAs are often considered as a "fingerprint" of the tissue they were derived from. For this reason they have the potential to become instrumental biomarkers reflecting the pathophysiological state of a tissue. Although still a relatively new field of study, miRNA analysis has been examined for disease diagnosis, prognosis, and evaluation of a treatment response. Circulating miRNAs are currently being used as biomarkers for cancer, cardiovascular disease, neurological disorders and other maladies [7-10]. In addition, there are several miRNAs that are associated with metabolic diseases such as insulin resistance, obesity and fatty liver disease. The following soluble miRNAs are prospective candidates for detecting early signals of drug efficacy in clinical studies.

Insulin Resistance and Type 2 Diabetes

Insulin resistance is the inability of insulin, a pancreatic hormone, to efficiently stimulate glucose uptake in peripheral tissue such as skeletal muscle, and is the hallmark of type 2 diabetes. Let-7 and miR-126 have been characterized as two key miRNAs associated with insulin resistance. Let-7 was the first miRNA identified in humans. It regulates glucose metabolism by directly targeting several members of the insulin signaling cascade [11]. Moreover, members of the Let-7 family are reduced in serum of type 2 diabetes subjects compared to healthy controls [12]. A yearlong dietary intervention significantly increased plasma Let-7b* levels 8-fold in pre-menopausal women [13]. Furthermore, anti-diabetic pharmacological treatment in naïve type 2 diabetes subjects showed a substantial increase in Let-7a and Let-7f levels [12]. Circulating miR-126 expression is also significantly reduced in type 2 diabetes subjects compared to control subjects [14]. Interestingly, miR-126 is associated with diabetes complications such as cardiovascular disease. To this end, miR-126 is highly expressed in heart tissue and has been shown to play a role in heart disease through regulation of angiogenesis and inflammation (reviewed in [10]).

On the other hand, miR-140-5p, miR-142-3p and miR-222 are all increased with type 2 diabetes [15], as well as morbid obesity [16]. Furthermore, a 3-month metformin treatment intervention in type 2 diabetes subjects was associated with a significant reduction in plasma miR-140-5p and miR-222, as well as increased miR-142-

3p and miR-192 levels [15]. Interestingly, miR-192 was recently identified as a prediabetes maker [17], a metabolic condition associated with high risk of developing type 2 diabetes. Finally, at the level of the pancreas, miR-375 is the most abundant miRNA and has been shown to regulate insulin secretion [18]. miR-375 expression is increased in the pancreas of type 2 diabetes subjects [19] and is also elevated in serum of newly diagnosed type 2 diabetes subjects compared to controls [20].

Both lifestyle and pharmacological treatment of diabetes can impact miRNA patterns [21], together with the fact that miRNAs are associated with different stages of diabetes progression, and play a pivotal role in the disease etiology, miRNAs are poised to become meaningful pharmacodynamic biomarkers.

Obesity

Excess fat accumulation results in an increased body mass index (BMI) and obesity. miR-520c-3p and miR423-5p both negatively associate with several indices of obesity such as BMI, percent fat mass and waist circumference. Moreover, these two miRNAs are significantly lower in morbidly obese than obese or overweight subjects [16]. Interestingly, miR423-5p expression is altered upon weight loss interventions, bariatric surgery significantly increased miR423-5p levels [16]. Another obese-centric miRNA is miRNA-27. High glucose concentrations stimulates miR-27a secretion from fat cells [22] and this miRNA is also thought to regulate fat cell expansion [23]. Interestingly, blood miR-27a concentrations correlate with fasting glucose levels and miR-27a levels are elevated in subjects with type 2 diabetes and metabolic syndrome (a metabolic condition associated with central obesity and cardiovascular disease) [24]. In addition, circulating miR-27 is significantly higher in obese children compared to age- and sex-matched lean controls [25]. Altogether, miRNAs can distinguish between severity of obesity as well as diabetes, making them a versatile indicator of metabolic disease progression, which is a highly desirable element of a biomarker.

Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver dysfunction associated with hepatic steatosis (fat accumulation), and nonalcoholic steatohepatitis (NASH) represents a more severe form of the disease resulting in liver inflammation and injury as well as steatosis. Currently NASH is diagnosed and a treatment response is evaluated by painful liver biopsy procedures, therefore there is imperative need for the development of a standardized non-invasive biomarker for this indication. In this respect, miR-122 expression is hepatic-specific and is the most abundant miRNA identified in the liver (reviewed in [26]). Animal studies suggest that miR-122 is a key regulator of cholesterol and triglyceride metabolism (reviewed in [27]). In humans, plasma miR-122 levels are increased in NAFLD, correlate with NASH biopsy measurements, and are able to distinguish the degree of NAFLD severity [28]. Moreover, as a liver-specific biomarker, miR-122 has also been investigated as an indicator of drug-induced liver injury [29]. Therefore, miR-122 monitoring has the potential to be extremely useful in clinical studies as a gauge of liver impairment and to ascertain a therapeutic response.

Power in Profiling

Other miRNAs of interest associated with metabolic diseases such as type 2 diabetes, obesity and NAFLD include miR-15, miR-21, miR-33, miR-34a, miR-103/107 and miR-451 [26, 27, 30, 31]. Detailed lists of miRNAs and their gene targets have been compiled into databases and online resources (e.g. miRBase [32] and miRWalk [33]). While all the miRNAs mentioned above show strong association with a metabolic disease state; miRNA profiling, a platform examining several miRNAs at once, can yield more robust results. Numerous studies report that miRNA signatures or distinct patterns appear tissue specific for various disease conditions (reviewed in [27, 30, 34]).

Quantitation of miRNA

miRNA can be extracted from a wide range of sources including whole blood, plasma, saliva, tears, urine, amniotic fluid, bronchial lavage, cerebrospinal fluid, stool specimen, cell lines, fresh tissues, and formalin-fixed paraffin-embedded (FFPE) tissues. The first step in miRNA quantitation is to select the appropriate sample as the miRNA expression varies significantly among sample sources. Moreover, the extraction site (total vs. micro-vesicle specific) can also impact the results (reviewed in [8, 21]). Contamination in serum or plasma by miRNAs from blood cells is also a significant concern. Therefore it is imperative that plasma/serum preparations be free of hemolysis.

While miRNA analysis techniques have not yet reached the same level of maturity as the most routine methods for RNA and DNA analysis, there are several existing, robust techniques available for the analysis of miRNA. The most commonly used techniques are RT-qPCR, microarrays and Next Generation Sequencing (NGS). The advantages and disadvantages of these three techniques are outlined in Table 1.

Table 1: Advantages and Disadvantages of miRNA analysis techniques

Methodology	Advantages	Disadvantages
RT-qPCR	Quantitative High sensitivity Broad dynamic range Can be highly specific when the primers are designed appropriately	Cannot identify novel miRNA Amplification required Specificity is dependent on primer design
Microarray	High throughput multiplexing Multiple vendors/platform Moderate sensitivity	Low specificity Cannot identify novel miRNA
NGS (Next Generation Sequencing)	Identification of new miRNA High sensitivity and specificity	High cost Long run time

RT-qPCR is considered the gold standard for miRNA quantitation in a regulated environment due to its high sensitivity, specificity, and broad dynamic range [35, 36]. Prior to performing RT-qPCR analysis, it is important to isolate high quality RNA using a robust and reproducible method. The quality of total RNA should be evaluated by spectrophotometry and a microchannel-based electrophoretic cell instrument, such as Agilent Bioanalyzer and Bio-Rad Experion system. In some cases exogenous spikes of synthetic miRNAs can be added before extraction and measured in the purified RNA to monitor the quality of the extraction.

The first step in RT-qPCR is the reverse transcription of miRNA to cDNA. Reverse transcription primers designed specifically for miRNA are used for this purpose. The primers are designed to have a short single sequence complementary to the 3' end of the miRNA, followed by a double stranded loop containing the universal primer sequence. The presence of the stem loop in the primer ensures the detection of the mature form of the miRNAs instead of their precursors. The 3' and 5' ends of primers are designed to complement the target miRNA and enable reverse transcription. Alternatively, miRNAs can be tailed by polyadenylation at their 3' end. The oligo-dT primers are used for reverse transcription. Various approaches such as incorporation of locked nucleic acids (LNAs) into the primer design can be used to increase specificity and sensitivity of qPCR.

One of the challenges in miRNA quantitation, regardless of the technology used, is the appropriate data normalization. A frequent approach for normalizing data is the use of invariant endogenous controls. An ideal endogenous control should exhibit minimal biological variation in the system under investigation. It should also have sufficient expression and be stable under study conditions for robust quantitation. RNAs such as 5S, U6, 18S etc. has been reported as normalization controls, however the justification for using them are still lacking [37].

The analytical approaches used in miRNA quantitation are still a relatively new field of study. More cross-platform confirmation and validation studies are needed to address the challenges mentioned here. The analytical aspects of miRNA profiling have a substantial impact on subsequent biological interpretations and their translational applications.

Considerations for Early Clinical Studies

The biotech industry has positioned miRNAs as an attractive therapeutic candidate through inhibition or mimicking function, as well as biomarkers for disease. Drug development programs aiming to treat a metabolic disease may benefit from the assessment of miRNAs as they are often causal to the disorder, altered in a diseased state and influenced by a therapeutic intervention. Due to their soluble nature, miRNAs are ideal biomarkers however, before including these measurements in a clinical study, the following ethical and sample collection recommendations should be considered. Since miRNA determination is a form of genetic testing, specific informed consent must be obtained for these analyses and sample retention. Furthermore, sample acquisition (fasting or fed conditions), processing (tube preservatives), and storage conditions are key considerations to maximize sample recovery and minimize variability. A laboratory subject matter expert can provide guidance on these factors as well as suggest strategic miRNAs for a particular drug development program.

Conclusion

The dawn of the "omics" field has led to comprehensive metabolic screening at the level of the genome, proteins, metabolites and now miRNAs. Several miRNAs have emerged as soluble biomarkers of metabolic diseases, and as research in this growing domain continues, it is expected that more will arise. By providing insight into that status of a specific cell or tissue, soluble miRNA biomarkers have the potential to make a significant impact in clinical research as an early signal of drug efficacy.

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