Clinical Chemistry Trainee Council
Pearls of Laboratory Medicine
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TITLE: Introduction to miRNA

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Slide 1:
Hello, my name is Alicia Algeciras-Schimnich. I am co-director of the Endocrine and Automated Immunoassay Laboratories at Mayo Clinic and Assistant Professor of Laboratory Medicine and Pathology at Mayo Clinic College of Medicine. Welcome to this Pearl of Laboratory Medicine on "Introduction to microRNA."

The use of microRNA as disease biomarkers is an emerging area in clinical diagnosis. Here at Mayo Clinic, the Endocrine laboratory is investigating the utility of miRNAs as biomarkers for thyroid cancer.

Slide 2:
microRNAs (miRNAs) are a class of endogenous non-protein coding RNA sequences of approximately 18–24 nucleotides long that regulate gene expression, thereby controlling many cellular processes including cell growth, differentiation, proliferation, and apoptosis. The specificity of miRNAs is dictated by only six to seven nucleotides of the 18-24 nt sequence. Therefore, a single miRNA can potentially target hundred of genes. Since the discovery of the first miRNA in the worm *C. elegans* in 1993, alterations in miRNAs expression have been correlated with disease pathogenesis. A number of diseases have been shown to have altered miRNA expression including multiple cancers, cardiovascular disease, and Alzheimer's disease.

Slide 3:
miRNAs are mostly contained within the introns of protein coding genes. In the nucleus, miRNAs are transcribed by a RNA polymerase II or III into a poly stem-loop structure. This structure is subsequently cleaved by the RNAse III enzyme DROSHA to single stem-loop structures of ~ 70 nucleotides that are then exported to the cytoplasm by the nuclear export protein Exportin 5.

Slide 4
miRNAs are further processed in the cytoplasm by another RNAse III enzyme, Dicer, resulting in a 18-24 double stranded duplex. After unwinding of the duplex, the mature miRNA (shown in yellow) is incorporated into the miRNA-induced silencing complex or RISC. This complex regulates gene expression by binding to complementary mRNA sequences. This binding either results in repression of translation if

not completely complementary to the mRNA, or cleavage and degradation of the mRNA if complementation is perfect. miRNA is thus a fine-tuning mechanism by which cells can control gene expression beyond the standard transcription-translation paradigm. It is estimated that 1/3 of all human genes are regulated in this manner.

Slide 5:

Given that miRNAs modulate mRNAs, you might wonder why not simply examine the mRNAs themselves? There are some advantages for analyzing miRNAs instead of mRNA. The most significant, miRNAs are far more stable of an analyte than mRNAs. Part of this superior stability has been attributed to the short sequence of the miRNAs in comparison to the mRNA. miRNAs has been detected and successfully extracted from multiple tissues such as fresh frozen tissue, paraffin-embedded formalin fix tissues, and fluids including serum, plasma, saliva, urine, amniotic fluid, and CSF. Finally, there are a limited number of miRNAs in the human genome. The current release of miRBase - the central repository of all miRNA sequences - contains 1,852 human precursors miRNA and 2,578 mature miRNA sequences (as of 7/11/2013; www.mirbase.org), compared to tens of thousands of mRNAs, making profiling of miRNAs more manageable.

However, there are also limitations or disadvantages when trying to use miRNAs in clinical laboratories. First, they are present in low concentration compared to other nucleic assays. This is particularly problematic when isolating miRNAs from body fluids where the amount of miRNAs obtained will be at very low concentration, making reliable quantitation challenging. In fact, when working with miRNAs in serum or plasma, traditional spectrophotometric quantification is not possible and instead, input is usually normalized to the initial volume of serum or plasma used for extraction. There is also lack of analytical and clinical validation since most published studies are focused on proof of principles studies and used a limited number of samples. Lack of standardization of miRNAs extraction and quantitation techniques represents a critical obstacle to clinical implementation of miRNAs as disease biomarkers. From the clinical perspective, association between miRNAs and patient outcomes will require well-designed prospective, blinded clinical studies.

Slide 6:

Current approaches for miRNA analysis include deep sequencing, microarrays, and real-time polymerase chain reaction (PCR), also called *quantitative real time polymerase chain reaction*. The use of deep sequencing allows for discovery of new miRNAs as well as identification of single nucleotide polymorphisms (SNP) within the miRNA sequence. On the other hand, microarray and real-time PCR assays have a predetermined number of miRNAs and are often used to evaluate the differential expression of a known subset of miRNAs in a particular disease. As shown in this table, there are differences between each technology in the context of template requirements, assay reproducibility, data complexity, and cost that needs to be taken into consideration when designing miRNAs studies. In the clinical laboratory, the most amenable technique for routine use will likely be real time-PCR assays.

Slide 7:

The interest in using miRNAs as disease biomarkers has been strengthened by their discovery in circulation. The first evidence that the miRNAs could serve as serological biomarkers of solid tumors was provided by Mitchell et al and Chen et al.

Mitchell et al demonstrated that miRNAs that are present in human plasma or serum are remarkably stable after incubation of the specimens at room temperature of up to 24 hours and resistant to multiple freeze-thaw cycles. This group also reported that serum levels of miR-141 were frequently overexpressed in prostate cancer patients when compared to healthy controls.

Chen et al used Solexa sequencing to analyze the repertoire of serum miRNAs in healthy individuals and patients with lung cancer, colorectal cancer, and diabetes. Using this approach, they identified specific expression patterns of serum miRNAs for lung cancer, colorectal cancer, and diabetes, providing evidence that serum miRNAs contain fingerprints for various diseases.

Both of these studies established the proof-of-principle for using circulating miRNA signatures as minimally invasive disease biomarkers. Since these original studies, altered expression of miRNAs in circulation has been described for a number of conditions such as cancer, cardiovascular disease, and Alzheimer's disease, to mention some examples.

Slide 8:
One area that is actively been investigated is how miRNAs are released into the bloodstream. Considering the resistance of miRNA to endogenous RNase activity, it has been postulated that it is unlikely that a significant fraction of cell-free miRNAs are passively leaked from cells as naked RNA molecules. In fact, synthetic miRNAs spiked into human serum or plasma are promptly degraded. Circulating miRNAs have been found to be packaged into various membrane-bound vesicles including exosomes, microvesicles, and apoptotic bodies and to exist in vesicle-free form associated with RNA-binding proteins or high-density lipoprotein complexes.

Slide 9:
There are a number of variables that might affect the interpretation of circulating miRNAs in clinical practice. For example, hemolysis might result in increased levels of miRNA in serum or plasma, if a particular miRNA is present at high levels in erythrocytes. The specimen type, for example plasma versus serum, might also influence interpretation depending on the degree of cellular contamination present in the preparation. Another variable to consider is how to adjust for template input, meaning whether a constant RNA concentration versus a constant volume is used for normalization of the miRNAs results.

Slide 10:
This graph further explains the effect of hemolysis in the quantification of four miRNAs. When serum is spiked with a RBC hemolysate to obtain hemoglobin concentrations between 25 and 1200 mg/dL, hemolysis had a significant effect on miR-16 levels. In this case, a change of 2 quantification cycles (Cq), representing a 4-fold change in miRNA concentration, was observed with as little as 25 mg/dL hemoglobin. Grossly hemolyzed samples had up to a 7 Cq change, representing a 128-fold change in miRNA concentration. miR-24 was also affected by hemolysis, though to a lesser degree. A change of >2.0 Cq was not observed until the hemoglobin concentration had reached 600 mg/dL. A third endogenous miRNA, miR-122, was not significantly affected by hemoglobin concentrations up to 1200 mg/dL. The exogenously added synthetic *C. elegans* miRNA was not affected by hemolysis, indicating that the changes observed for miR-15b, miR-16, and miR-24 are likely due to the release of these miRNAs from erythrocytes and not a direct effect of hemoglobin or other erythrocyte components on

the miRNA extraction, RT, or amplification process. This is important because if changes in miRNA expression are used to distinguish between the absence or presence of a disease, the presence of hemolysis might artificially bring the expression of the miRNA above a selected clinical cut-off.

Slide 11:
Another variable to consider when interpreting circulating miRNAs profiling studies is the effect of different specimen types. Serum, plasma, and whole blood have been studied as sources of circulating miRNAs. Serum has been described to have a lower amount of circulating miRNAs than plasma. This difference has been attributed to the presence of cellular contaminants derived from platelets in plasma. On the other hand, compared to plasma and serum, cells have much higher concentrations of miRNAs, which may lead to confounding results if adequate care is not taken during blood processing; specifically, different centrifugation protocols might result in different degree of cellular material removal. To minimize platelet contamination, it has been suggested that serum samples might be a preferable specimen type.

Slide 12:
Regardless of whether miRNAs are evaluated in body fluids or tissue specimens, there are multiple potential applications of miRNA in clinical practice. As disease biomarkers, miRNAs might be used in diagnosis by comparing the level of expression of one or a panel of miRNAs to distinguish between the absence or presence of a disease. In prognosis, the differential expression of miRNA might be indicative of better or worse disease outcomes. For example, increased expression of miR-196a-2 has been suggested to predict poor survival in patients with pancreatic cancer. For therapy, differential expression of miRNA might be useful to predict drug efficacy. In breast cancer patients, it has been suggested that increased expression of miR-221 and mir-222 might predict tamoxifen-resistance in breast cancer.

Beyond their value as diagnostic markers, a role for miRNAs in the pathogenesis of various diseases highlights their potential as targets for therapy. The goal in therapy will be to modulate miRNA expression by either re-established expression of silenced miRNAs or silencing the expression of overexpressed miRNAs. One such example is the regulation of miR-122 for the treatment of Hepatitis C virus (HCV) infection. miR-122 is a miRNA expressed in the liver and is essential to the stability and propagation of HCV RNA. Miravirsen is a locked nucleic acid–modified DNA antisense oligonucleotide that sequesters mature miR-122, thereby inhibiting its function. The outcomes of Phase 2 trial evaluating the safety and efficacy of miravirsen have recently been published. The use of miravirsen in 36 patients with chronic HCV genotype 1 infection showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance.

Slide 13:
A great deal of research has been done in the utility of miRNAs in cancer. This table shows an example of the numerous miRNAs that have altered expression in malignant tissue. Of interest is the fact that in many cancers, at least one miRNA seems to be commonly upregulated or downregulated, which is consistent with the role of miRNAs in controlling multiple cellular processes including those leading to cell transformation and tumorigenesis. For example, miR-21 has been reported to be upregulated in multiple cancers. Biologically, miR-21 has been shown to promote cell proliferation, migration, invasion, and metastasis. On the contrary, miR-145 is downregulated in various cancers consistent with its role as

a tumor suppressor. Therefore, if considering miRNAs as potential diagnostic markers for cancer, it is necessary to bear in mind that some miRNAs might not be specific for a particular cancer but representative of the processes leading to malignant transformation.

Slide 14:

There are a limited number of commercially available tissue miRNA assays to aid in the diagnosis of malignancies. miR*Inform*® Pancreas (Assuragen) measures the expression levels of seven miRNAs via reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The test could be used as an aid in the diagnosis of pancreatic ductal adenocarcinoma (PDAC) in fine needle aspirate biopsy (FNAB) specimens. A proprietary algorithm is applied to generate a score between 0 and 1 (inclusive). A score less than 0.5 is considered benign and a score between 0.5 and 1 is considered to be PDAC. When this test was applied in FNAB samples with indeterminate and benign FNA cytology, a sensitivity of 94% and specificity of 83% were reported.

The Rosetta Lung Cancer Test™ (Rosetta Genomics) measures the expression of eight miRNAs to discriminate between four main types of primary lung cancer: squamous cell carcinoma, non squamous cell carcinoma, small cell carcinoma, and lung carcinoid tumors. The test could be performed in formalin-fixed paraffin embedded tissue, FNAB, and Bronchial Brushings. A sensitivity of 94% and specificity of 98% have been reported for this assay.

Slide 15:

miRNAs are small nucleotide single-stranded non-protein coding RNAs that act as post-transcriptional regulators of gene expression. miRNAs represent emerging biomarkers with a wide range of potential clinical applications, including diagnosis, assessment of prognosis, prediction of treatment efficiency, and potential therapeutic applications. A number of studies have established the proof-of-principle on the use of miRNAs but additional work is needed to validate the miRNAs/disease association in independent studies. miRNA biomarkers in tissue have reached the clinical diagnostic setting; however, the application of these biomarkers in serum or plasma represents bigger challenges.

Slide 16: References

Slide 17: Disclosures

Slide 18: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on “Introduction to miRNA.” I am Alicia Algeciras-Schimnich.