Identifying Circulating MiRNAs as Potential Biomarkers for Kidney Disease by qRT-PCR

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Abstract

MicroRNAs (miRNAs) are short endogenous non-coding RNA molecules that play critical roles in gene regulation, cell differentiation, proliferation, apoptosis, and metabolism. MiRNA levels in blood and other body fluids are altered in many disease stages and may be potential biomarkers for diagnosis and prognosis. Evaluation of miRNA from these matrices is challenging due to the low abundance of nucleic acids which can impact extraction efficiency and reproducibility. To explore the utility of miRNAs as clinical biomarkers in human serum and urine, I developed an assay procedure using Trizol-LS for extractions of serum or urine in the presence of glycogen carrier. This extraction method resulted in the efficient and reproducible recovery of a spiked *C. elegans* miRNA control, cel-miR-39, as well as two endogenous miRNAs, hsa-miR-16 and hsa-miR-223, using specific Taqman miRNA Assays. With this optimized technique, a list of miRNA targets were obtained from a screening assay that compared serum and urine collected from healthy normal donors and patients with proteinuria or nephrotic syndromes. A few miRNA targets with the greatest alteration in expression level between normal and disease groups were further evaluated using individual Taqman Assays. MiR-146b appeared to be significantly down-regulated in serum from the disease group, while miR-21 seemed slightly up-regulated in nephrotic urine. Overall, the method developed in this thesis provides a robust approach to isolate and detect circulating miRNA in human serum and urine with high reproducibility. This study also suggests a

list of miRNA candidates that are worth further investigation as potential non-invasive biomarkers for kidney related diseases.

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Chapter I

Introduction

Kidney and kidney-related diseases are one of the most prevalent disease categories affecting millions of patients worldwide (Boger & Heid, 2011). The current gold standard to diagnose complex kidney disorders comprises a series of blood tests combined with kidney biopsies which are both invasive and error-prone (Tesch, 2010). Blood tests can assess kidney function by measuring blood biomarkers such as blood urea nitrogen and serum creatinine; however, challenges for developing such protein-based biomarkers are enormous due to the complex proteomics in blood, the diversity of post-translational modifications and the relatively low abundance of these molecules (Etheridge, Lee, Hood, Galas, & Wang, 2011). Therefore, seeking alternative biomarkers to diagnose kidney diseases is of great importance to help prevent disease progression through early treatment, which, in consequence, will not only save human lives but tremendously reduce national healthcare cost.

Kidney and Kidney Related Diseases

Kidneys are a pair of bean-shaped organs that lie on either side of the spine in the lower middle of the back. Each kidney contains approximately one million filtering units called nephrons, each of which is made of a glomerulus and a tubule. The glomerulus is a

miniature filtering device while the tubule is a small tube-like structure attached to the glomerulus (Smith, 1955). Kidneys function to remove waste products and water from blood. They also play critical roles in regulating levels of various elements in the blood such as calcium, sodium and potassium (Li, Yong, Michael, & Gleadle, 2010).

Chronic kidney disease (CKD) is defined by gradual and usually permanent loss of renal functions over time. CKD is a common disease affecting 10% of the general population, and causing significant morbidity and mortality (Boger & Heid, 2011). The disease status of CKD is often classified into five stages with increasing severity. Patients with CKD usually start with minimal symptoms that are mostly unnoticed (stage 1) and will gradually progress to more severe stages if not treated immediately. At stage 5, the end stage renal disease, it is so severe that the kidney loses its function completely. Symptoms of chronic kidney disease vary from mild ones such as frequent urination, fatigue and weakness to severe ones such as high blood pressure and chest pain. With loss of kidney function, water, waste and toxic substances, which are normally excreted by the kidney, now accumulate in the body. Kidney malfunction is also associated with a variety of other problems such as anemia, high blood pressure, disorders of cholesterol and fatty acids, and bone diseases. The two main causes of CKD are diabetes and high blood pressure (Boger & Heid, 2011). In addition, recent genome-wide studies provide novel insights into the development of CKD at the genetic level. Genome-wide association studies (GWAS) provide a powerful hypothesis-free approach to identify associations between CKD and a panel of several million genetic variants distributed across the genome. UMOD, SHROOM3, STC1, LASS2, GCKR, ALMS1, TFDP2, DAB2, SLC34A1, VEGFA, PRKAG2, PIP5K1B, ATXN2/SH2B3, DACH1, UBE2Q2,

and SLC7A9 were uncovered as loci associated with estimated glomerular filtration rate and CKD (Boger & Heid, 2011), suggesting that both genetic and environmental factors play a role in disease development.

Nephrotic syndrome describes a series of symptoms caused by damage to the glomeruli of the kidney, which result in leakage of large amounts of protein from the blood into the urine. One example of nephrotic syndrome is Focal Segmental Glomerulosclerosis (FSGS), which accounts for approximately 20% of cases of the nephrotic syndrome in children and 40% of such cases in adults (D'Agati, Kaskel, & Falk, 2011). Although the specific causes of FSGS are unknown, it appears to be associated with mutations in specific podocyte genes that encode nephrin and podocin (D'Agati et al., 2011). The major symptom of FSGS is nephrotic proteinuria, which results from loss of integrity of the glomerular filtration barrier. The glomerular filtration barrier (GFB) is a highly specialized blood filtration interface that is freely permeable to water and small- and midsized solutes in plasma, yet maintains considerable size and charge selectivity for proteins and larger molecules. This barrier has three major components: the glomerular endothelial cell, the basement membrane and visceral epithelial cell (podocyte). In FSGS patients, injured podocytes lead to the effacement of podocyte foot processes, which results in podocyte depletion into urine (Ho et al., 2008). Loss of more than 40% of podocytes leads to FSGS with high grade proteinuria and renal insufficiency (Wharram et al., 2005). In the United States, FSGS is the most common primary glomerular disorder causing end-stage renal disease with a prevalence of 4% (D'Agati et al., 2011). So far, the diagnosis of FSGS relies mainly on invasive biopsy after symptoms of disease are clinically significant. Identification of potential FSGS-associated urinary biomarkers

representing pre-sclerotic and serial sclerotic stages of FSGS could be helpful as a non-invasive screening tool for diagnosis and for monitoring prognosis of FSGS (Shui et al., 2008). For instance, sclerosis of glomeruli is the key determinant of FSGS; proteins that appear in the urine before the onset of glomerular sclerosis would have higher diagnostic value as early biomarkers than those appearing after the onset.

MicroRNAs

Similar to CKD and FSGS, an increasing number of kidney-related diseases appear to be linked to disease-associated variants in human genome. Novel approaches for disease classification, detection and diagnosis using genetic tools have emerged rapidly in recent decades with the help of advancing new technology. A series of molecular markers such as messenger RNAs (mRNAs) and microRNAs (miRNAs) have joined the family of biomarkers which have long been dominated by proteins such as cytokines and antibodies.

Small RNA molecules that regulate gene expression were first described in 1993 by Lee and his colleagues (Lee, Feinbaum, & Ambros, 1993), and the term microRNA was introduced in 2001 (Ruvkun, 2001). MiRNAs are small non-coding regulatory RNAs 20-22 nucleotides in length. These conservative small RNA molecules play critical roles in gene regulation by binding primarily to complementary sequences in the 3'-untranslated regions (3'-UTR) of specific mRNAs (Chen, Tan, Wong, Fekete, & Halsey, 2011). Although the human genome only contains approximately 1000 miRNAs, they are

believed to impact almost every cellular pathway (Chen et al., 2011). Recent evidence suggests that miRNA levels change with disease or injury (Starkey Lewis et al., 2011). Lu et al. (2005) showed that the classification of poorly differentiated tumors by miRNA-profiling was more accurate than the use of mRNA classifiers, suggesting a potential role of miRNAs in detection and diagnosis of cancer (Lu et al., 2005). In addition, studies demonstrate that miRNAs are present in human plasma and serum in a remarkably stable form that is protected from endogenous RNase activity (Mitchell et al., 2008). Other studies suggested that miRNAs may serve as biomarkers in a number of diseases including Alzheimer's disease (Geekiyanage, Jicha, Nelson, & Chan, 2011) and drug-induced liver injury (Starkey Lewis et al., 2011).

Although the function of miRNA in kidney disease is poorly understood, Hanke and his colleagues identified elevated levels of miR-126 and miR-182 in the urine of patients with urinary bladder cancer (Hanke et al., 2010), suggesting kidney-specific miRNAs which are present in urine may provide insight into the health of the kidney.

A recent publication demonstrates that miR-21 promotes kidney fibrosis in two mouse models. Chau et al discovered that in mice, miR-21 was up-regulated in the kidney soon after injury, before fibrosis appears (Chau et al., 2012). Consistent with the findings in mice, miR-21 is up-regulated in human kidneys from patients with problems such as acute kidney injury. Analysis of gene expression study suggested that miR-21 regulates groups of genes in several metabolic pathways. These studies indicate that miR-21 plays a critical role in fibrogenesis and epithelial injury in the kidney and may become the first therapeutic target for antifibrotic therapies. The circulating level of miR-21 in blood and

urine was not analyzed in these recent studies; however, this study suggests that miR-21 merits further analysis as a potential disease biomarker.

MiRNA Quantitation

Accurate quantitation of miRNA levels is vital for the analysis of miRNA regulation and function. An accurate and reproducible method for extraction, detection and quantitation of miRNA is the first step for any down-stream analysis. The current gold standard for miRNA quantitation is qRT-PCR; a number of qRT-PCR based methods have been introduced since 2004 (Chen et al., 2011). The most commonly used methods are TaqMan microRNA assays and arrays developed by Life Technologies. The advantages of TaqMan assays include high sensitivity and specificity, large dynamic range, and reputable results validated by peers in laboratories across the world. The Taqman Array miRNA Card is a 384-well megaplex platform that enables the simultaneous detection of 377 miRNAs from a single reverse transcription reaction, greatly reducing the amount of starting material and the number of RT reactions required for quantitative gene expression analysis. MiRNA Taqman Assays, on the other hand, are singleplex qRT-PCR kits focusing on a single miRNA target for further quantification. The TaqMan Assay, used in both platforms, is a real time PCR technique that uses a pair of unlabeled PCR primers and a TaqMan probe with a FAM or VIC dye label on the 5' end, and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3' end. The technique relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence resulting in

fluorophore-based detection. RNA from the sample of interest is reverse transcribed into cDNA, and the synthesized cDNA serves as the template for real-time PCR. The resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR. As compared to other real-time PCR methods, the TaqMan probe significantly increases the specificity of the detection. Other than Life Technologies, a number of smaller vendors such as Exiqon also provide full kits for miRNA research, but the validity and comparability of these assay kits need further confirmation.

Extraction and purification of miRNA is a big challenge because of their short length, sequence similarity and low abundance in human fluid such as serum and urine. For serum samples, several purification methods have been proposed in recent publications. The traditional methods of RNA purification include Trizol and chloroform, chemicals that can separate RNA, DNA and protein into different layers but are highly toxic if contacted directly by skin. Other commercially available miRNA purification kits are mostly column-based including Qiagen's miRNeasy Kit and ABI's mirVana miRNA Isolation Kit.

Only a limited number of published studies focus on miRNA in urine, possibly because of its high content of contaminants and person-to-person variations. But with advancing technology, urine has emerged as a new area of interest. A paper published last year summarized the data in recovering circulating extracellular or cell-free RNA from various bodily fluids including urine (Tzimagiorgis, Michailidou, Kritis, Markopoulos, & Kouidou, 2011). In a recent study, Hanke and his colleague (2010) developed a robust method to study urine miRNA as tumor markers for urinary bladder cancer. They

prepared urine samples using the miRNeasy kit (Qiagen) and RNA extraction with phenol/chloroform followed by Taqman miRNA analysis (Life Technologies) (Hanke et al., 2010). Results showed that a number of miRNAs were significantly up-regulated in the urine samples collected from patients with urinary bladder cancer, suggesting a correlation between urinary miRNA levels and disease status. These results suggest that miRNA levels in urine may change in the context of other kidney diseases including CKD and FSGS, offering a non-invasive method for disease diagnosis and monitoring disease progression. In the study described here, I have optimized a technique for extraction of miRNA from urine and serum and have identified several miRNA markers that may be associated with kidney disease.

Chapter II

Materials and Methods

Sample Collection and Storage

Eleven normal serum samples and six normal urine samples were either collected from Bioreclamation or collected from Genzyme donors as controls (Table 1). Due to limited sample availability, I selected six serum samples and three urine samples from Bioreclamation from patients with proteinuria or nephrotic syndromes (Table 2). Although the patients share a diagnosis of proteinuria or nephrotic syndrome, all of the serum samples were collected from individuals with type II diabetes and most also have hypertension. All serum and urine samples were aliquoted into 1.5mL RNAase Free tubes (Part No. AM12450, Ambion) and stored at $\leq$-60^{o}C immediately upon arrival or collection.

Table 1: List of normal samples used in this study.

ID	Description	Gender	Race	Age
BRH603164	Serum	Male	Black	26
BRH603165	Serum	Male	Black	45
BRH603166	Serum	Male	Black	23
BRH603167	Serum	Male	Hispanic	73
BRH603168	Serum	Male	Hispanic	41
BRH603169	Serum	Male	Hispanic	57
BRH615102	Serum	Female	Hispanic	37
BRH615103	Serum	Female	Black	20
BRH615104	Serum	Male	Hispanic	40
BRH615105	Serum	Female	Black	40
BRH615106	Serum	Male	Black	31
BRH576271	Urine	Male	Caucasion	39
BRH576272	Urine	Male	Caucasion	20
BRH576329	Urine	Female	Caucasion	52
Donor1	Urine	Male	Caucasion	40
Donor2	Urine	Female	Caucasion	45
Donor3	Urine	Male	Caucasion	36

Table 2: List of disease samples used in this study.

UID	Description	Gender	Medications	Diagnosis	Race	Age
92776	Serum	Male	Zofran,Clonazepam,Tekturna,Ambien,Lipitor, Coreg,Lorazepam	Type 2 Diabetes,Actinic keratosis,Anemia,Anxiety,B12 deficiency,Hypertension,Chronic Hypertrophic Gastritis,Constipation,Coronary Artery Disease,Depression,Type 2 Diabetes,Hypertension,Fatigue,Renal pain,Anxiety disorder,GERD,Headache,Hematoma of leg,Hyperlipidemia,Irritable Bowel Syndrome,Insomnia,Low back pain,Lumbar spinal stenosis,Mixed hyperlipidemia,Nausea,Muscle aches,Osteoarthritis,Polyneuropathy,Proteinuria,Vitamin D deficiency	Caucasian	67
37472	Serum	Male	Warfarin; Metoprolol; Celexa; Glipizide; Alendronate; Actos; Simvastatin; Stsrlix; Metformin; Humana pharm	Type 2 Diabetes; Atrial Fibrillation; Cellulitis of the arm; Chronic Kidney Disease; Coronary Artery Disease; renal manifestations; Hypertension; Hypercholesterolemia; Hypertrophy (benign) of prostate without urinary obstruction; low back pain; mixed hyperlipidemia; morbid obesity; old myocardial infraction; proteinuria; cardiac pacemaker; superficial arm vein phlebitis	Caucasian	78
72683	Serum	Female	Simvastatin, Lisinopril, Verapamil, Lantus, Cilostazol, Glipizide, Cipro, Metoprolol, Docusate Sodium, ASA, Zantac	Chronic Kidney Disease (stage 4),Type 2 diabetes, Hypertension (HTN), Hypercholesterolemia, Irritable Bowel Syndrome (IBS), Malignant Mixed Hyperlipidemia, Obesity, Old myocardial infarction, Osteoporosis, Proteinuria, Rheumatoid Arthritis	Caucasian	91
25214	Serum	Male	unknown	Chronic Kidney Disease; High Cholesterol; Type 2 Diabetes; Atherosclerosis; HTN	Caucasian	80
36490	Serum	Male	Atenolol; Lisinopril; Norvasc; Simvastatin; Ativan; Pantoprazole; Glipizide; Levothyroxine; One touch ultra lancets; Xalatan; Niacin; Zocor; Aspirin; Niacin	Type 2 diabetes; HypoT4; Aortic valve insufficiency; Chronic kidney disease; Chronic renal failure; HTN; Pulmonary HTN; HCE; Hypocalcaemia; MR; Hyperlipidemia; Myocardial infarction; Proteinuria; Sexual dysfunction	Caucasian	82
62071	Serum	Male	Plavix,Prednisone,Simvastatin,Viagra,Amlodi pine,Dulera	Type 2 Diabetes,Aortic stenosis,Coronary Artery Disease,Congestive Heart Failure, Chronic Kidney Disease,COPD,Dizziness,Emphysema,Erectile Dysfunction,Hypertension,Gastric Ulcer,Colonic Polyps,Hypertensive chronic disease,Low back pain,Lumbar Osteoarthritis,Hyperlipidemia,Muscle weakness,Pneumonia,Proteinuria	Caucasian	73
93648	Urine	Male	Zofran,Clonazepam,Tekturna,Ambien CR,Lipitor,Coreg,Lorazepam	Actinic keratosis,Anemia,Anxiety,B12 deficiency, Benign hypertension,Chronic hypertrophic gastritis,Constipation,Coronary Artery Disease,Depression,Type 2 Diabetes,Essential hypertension,Fatigue,Abdominal pain,GERD,Headache,Hematoma leg,HYpercholesterolemia,Hyperlipidemia,Irritable Bowel Syndrome,Insomnia,Irritatied seborrheric kerastosis,Lumbar spinal stenosis,Major depression,Mixed hyperlipidemia,Muscle aches,Nausea,Osteoarthritis,Polyneuropathy,Proteinuria,VItamin D deficiency	Caucasian	67
87956	Urine	Female	Folic Acid, Cellcept, Aspirin EC, Plaquenil	Lupus Nephritis, Proteinuria, Anemia	Black	32
67145	Urine	Male	Lipitor; Renvela; Nephrocap; Prilosec; Zemplar; Proscar	High Cholesterol; HTN; Nephrotic syndrome; Hyperparathyroidism; Chronic kidney disease (CDK)	Caucasian	50

Common kidney diagnoses are highlighted in red.

MiRNA Extraction/Purification

To optimize the extraction protocol, I evaluated a number of different extraction parameters including different extraction reagents, sample collection tubes, carrier molecules, and matrix concentrations. Using Trizol LS in place of regular Trizol for extractions of serum or urine mixed in RNase-Free 1.5mL Microfuge Tubes in the presence of glycogen carrier resulted in the efficient and reproducible recovery of a spiked *C. elegans* miRNA control , cel-miR-39, and two endogenous miRNA targets, hsa-miR-16 and hsa-miR-223, using target specific Taqman miRNA Assays. During RNA extraction, I took extra precautions including using dedicated RNA-only Biosafety Cabinet, cleaning surface with 10% bleach and RNaze-Zap wipes (Part No. AM9786, AM9788, Life Technologies), UV-crosslinking pipettes and sample racks before and after each assay to minimize potential contamination.

Trizol-LS Reagent (Part No. 10296010, Life Technologies) was used to isolate RNA from serum and urine samples. After several comparisons, I chose to use a starting sample volume of 200uL in RNase-Free 1.5mL Microfuge Tubes made by Ambion (Part No. AM12400, Life Technologies). The manufacturer's protocol was modified to include 25fmol spike-in cel-miR-39 control (Part No. MSY0000010, Qiagen), which was later used as a normalizer to control for extraction efficiency. In addition, 10µg glycogen (Part No. 10814-010, Life Technologies) was selected as a carrier to help form a pellet in the isopropanol precipitation step. The precipitated RNA pellets were air dried for 1 min and resuspended in 10uL nuclease free water.

MiRNA Screening

MiRNAs extracted from one normal and two disease serum samples were screened using the Taqman Array MicroRNA Card A v2.0 only (Part No. 4398965, Life Technologies). Two normal and two disease urine samples were screened using both Taqman Array MicroRNA Card A (Part No. 4398965, Life Technologies) and Card B v3.0 (Part No. 4444910, Life Technologies). The screening step was used to help select the miRNA targets with the largest difference in expression between normal and disease samples.

Extracted total RNA was first reverse transcribed to cDNA using High Capacity cDNA RT Kit (Part No. 4368814, Life Technologies) combined with Megaplex RT Primer pool (Part No. 4444750, Life Technologies), which contains a mixture of 384 primer pairs. To achieve optimal results, these cDNA products were initially pre-amplified using Taqman Pre-amp Mastermix (Part No. 4391128, Life Technologies) with Megaplex Pre-Amp Primer pool (Part No. 4444750, Life Technologies) before being amplified on the Taqman miRNA Array Card A or Card B. The cards, each containing 377 human miRNA targets, were run on ABI 7900HT real time PCR instrument and the data was analyzed using RQ Manager v1.2. An arbitrary threshold of 0.2 was used to determine the Ct values of each sample.

MiRNA Taqman Assay

For both serum and urine matrices, the singleplex miRNA Taqman Assay consists of a Reverse Transcription step and a qPCR step. Total RNAs isolated by Trizol were first reverse transcribed into cDNA using specific 5X miRNA assay primers (included in individual Taqman Assays ordered from Life Technologies, part numbers listed in Table 3) together with the High Capacity cDNA RT Kit (Part No. 4368814, Life Technologies). The cDNA was subsequently amplified in a qPCR assay using 20X miRNA assay primers (included in Taqman Assay ordered from ABI, part numbers listed in Table 3) and 2X Taqman Universal Mastermix (Part No. 4324018, Life Technologies). Taqman Assays were run on ABI 7900HT real time PCR instrument and data was analyzed with SDS v2.4. An arbitrary threshold of 0.2 was used to determine the Ct values of each sample.

Table 3: List of part numbers of Taqman miRNA Assays.

Taqman Assay	Part No. Life Technologies
miR-93	001090
miR-627	001560
miR-21	000397
miR-146b	001097
miR-451	001141
miR-629	002436
miR-642	001592
miR-1244	002791

Data Analysis

The miRNA screening results obtained by Taqman Array MiRNA card A and B on the ABI 7900 are presented as Ct values, which are inversely proportional to the expression level of targeted miRNA. Due to the lack of reliable endogenous controls, a mean-centering method was adopted to normalize all miRNAs on each card (Wylie, Shelton, Choudhary, & Adai, 2011). This method calculated the mean Ct value of all detectable miRNAs (Ct <35) within each sample, and used it as the normalizer to determine the ΔCt of each individual miRNA target (Wylie et al., 2011). The calculated ΔCt values of each miRNA target were then compared between normal and disease groups. The ones with the most significant expression difference were selected to be further evaluated using specific miRNA Taqman Assays.

Target specific Taqman Assays were analyzed using a different method of normalization. Within each sample, normal or disease, the Ct values obtained from each specific Taqman Assay were normalized based on the arithmetic mean of cel-miR-39, hsa-miR-16 and hsa-miR-223 to result in ΔCt. ΔCt of each individual sample was calculated by extracting the mean Ct values of the three normalization controls from the individual sample Ct. ΔΔCt was the difference between Ave-ΔCt_{normal} and Ave-$\Delta Ct_{disease}$. The resulting ΔΔCt values were used for miRNA expression analysis.

Method Validation

Extraction Precision – One serum and one urine sample were spiked with *C. elegans* miRNA (synthetic cel-miR-39, Part No. MSY0000010, Life Technologies) and extracted in eight individual extractions using Trizol-LS according to the established protocol. Extracted total RNAs were used as templates for RT (reverse transcription) using High Capacity cDNA Reverse Transcription kits (Part No. 4368814, Life Technologies). The level of the C. elegans spike and two endogenous miRNA targets, hsa-miR-16 and hsa-miR-223, were evaluated using Taqman Assays (cel-miR-39 Assay Part No. 000200; hsa-miR-16 Assay Part No. 000391; hsa-miR-223 Assay Part No. 002295, Life Technologies).

PCR Precision – One extracted serum and urine sample were evaluated for cel-miR-39 and hsa-miR-16 signals to test the PCR precision. Both samples were extracted using Trizol-LS. The extracted products were reverse transcribed into cDNAs in eight separate reactions using the High Capacity cDNA Reverse Transcription kit from Applied Biosystems. The resulting eight cDNA products were evaluated for cel-miR-39 and hsa-miR-16 signals using individual Taqman Assays.

Freeze-Thaw Stability – Serum samples were tested for freeze-thaw stability. One serum sample was extracted using Trizol-LS at baseline, and after each of five freeze-thaw cycles. Extracted total RNAs were carried through RT step using High Capacity cDNA Reverse Transcription kit and evaluated for hsa-miR-16 signals using a Taqman Assay.

Chapter III

Results

Method Validation

To ensure that the extraction and amplification methods used in this study generate reproducible results, I evaluated both extraction and PCR precision.

Extraction Precision – Analysis of eight separate extractions and amplifications of one serum and one urine sample which were spiked with *C. elegans* miRNA (cel-miR-39) indicate that the extraction procedure contributes some variability to the Ct results. The Ct values of cel-miR-39 and two endogenous targets (hsa-miR-16 and hsa-miR-223) from each extraction vary by 1.42-2.09 cycles indicating a maximum fold difference of 4.26 (Table 4). In serum, hsa-miR-16 exhibits the largest maximum Ct difference of 2.09; while in urine, hsa-miR-223 shows the greatest maximum Ct difference of 1.93.

Table 4: Summary of extraction precision results

	cel-miR-39					hsa-miR-16					hsa-miR-223				
	Ct	Mean Ct	Std Dev	%CV	Max Ct Diff.	Ct	Mean Ct	Std Dev	%CV	Max Ct Diff.	Ct	Mean Ct	Std Dev	%CV	Max Ct Diff.
Extraction Precision Serum	19.56	19.98	0.51	2.55%	1.46	28.73	29.82	0.63	2.11%	2.09	29.34	30.17	0.48	1.59%	1.42
	20.54					29.70					30.30				
	20.27					29.89					30.24				
	20.50					30.19					30.76				
	19.72					29.27					29.61				
	19.88					29.81					30.15				
	20.26					30.82					30.65				
	19.08					30.18					30.30				
Extraction Precision Urine	20.52	20.64	0.49	2.37%	1.42	34.34	34.83	0.62	1.79%	1.77	34.90	35.43	0.61	1.73%	1.93
	21.30					35.46					35.50				
	21.03					35.53					35.87				
	21.03					35.42					35.16				
	20.06					33.76					34.69				
	20.57					34.51					35.12				
	20.69					34.74					36.62				
	19.88					34.88					35.61				

%CV=(Std Dev) / (Mean Ct) * 100. Max Ct Diff. is the cycle difference between the samples with the greatest and smallest Ct values.

PCR Precision – Analysis of eight individual qRT-PCR reactions from a single serum and urine extraction indicate that qRT-PCR step contributes to the variability. The Ct values from each reaction for cel-miR-39 and the endogenous target hsa-miR-16 vary by 0.29-2.18 cycles indicating a maximum fold difference of 4.53 (Table 5). Hsa-miR-16 shows the largest variation with a maximum Ct difference of 0.69 in serum and 2.18 in urine.

The precision results indicate that Ct values may vary within the same sample by approximately 2 cycles for both serum and urine. These cycle differences define the background variability in this study; for a miRNA to be identified as differentially expressed it must exceed this level of difference.

Table 5: Summary of PCR precision results.

	cel-miR-39					hsa-miR-16				
	Ct	Mean Ct	Std Dev	%CV	Max Ct Diff.	Ct	Mean Ct	Std Dev	%CV	Max Ct Diff.
PCR Precision Serum	22.10	22.22	0.10	0.45%	0.29	31.43	31.15	0.25	0.82%	0.69
	22.20					30.91				
	22.21					30.90				
	22.06					31.08				
	22.25					31.14				
	22.35					30.92				
	22.31					31.59				
	22.28					31.20				
PCR Precision Urine	20.77	21.08	0.17	0.79%	0.55	35.31	35.33	0.72	2.04%	2.18
	21.02					34.83				
	21.17					34.49				
	20.94					35.02				
	21.13					36.14				
	21.32					35.15				
	21.14					35.01				
	21.14					36.67				

%CV = (Std Dev) / (Mean Ct) * 100. Max Ct Diff. is the cycle difference between the two samples with the greatest and smallest Ct values.

Freeze-Thaw Stability – To evaluate the stability of miRNA in serum which may undergo several freeze thaws prior to testing, I also examined the Ct value of the endogenous target hsa-miR-16 from three serum samples at baseline and after five freeze-thaws. Evaluation of the Ct values at all time points results in a %CV of 0.36 indicating a very small variation with increasing freeze thaw cycles. The max Ct difference between the six FT groups is 0.26 which is equivalent to a max fold difference of 1.2 (Table 6). These results are consistent with results from the literature that demonstrate that miRNA in serum is very stable (Mitchell et al., 2008).

Table 6: Summary of freeze-thaw stability results.

	FT base line	1 FT	2 FT	3 FT	4 FT	5 FT
Serum 1	29.12	28.79	28.94	28.31	29.17	28.80
	29.92	28.69	29.17	28.33	29.27	29.06
Serum 2	28.52	28.66	29.08	28.64	28.60	28.69
	28.46	29.39	28.97	29.08	28.66	28.94
Serum 3	28.69	28.93	28.92	28.70	28.30	28.88
	29.37	28.94	28.91	29.45	28.86	29.20
Ave	29.01	28.90	29.00	28.75	28.81	28.93
CV%	0.36%					
Max Ct Diff.	0.26					
*FT = Freeze Thaw						

%CV = (Std Dev) / (Mean Ct) * 100. Max Ct Diff. is the cycle difference between the time points with the greatest and smallest Ct values.

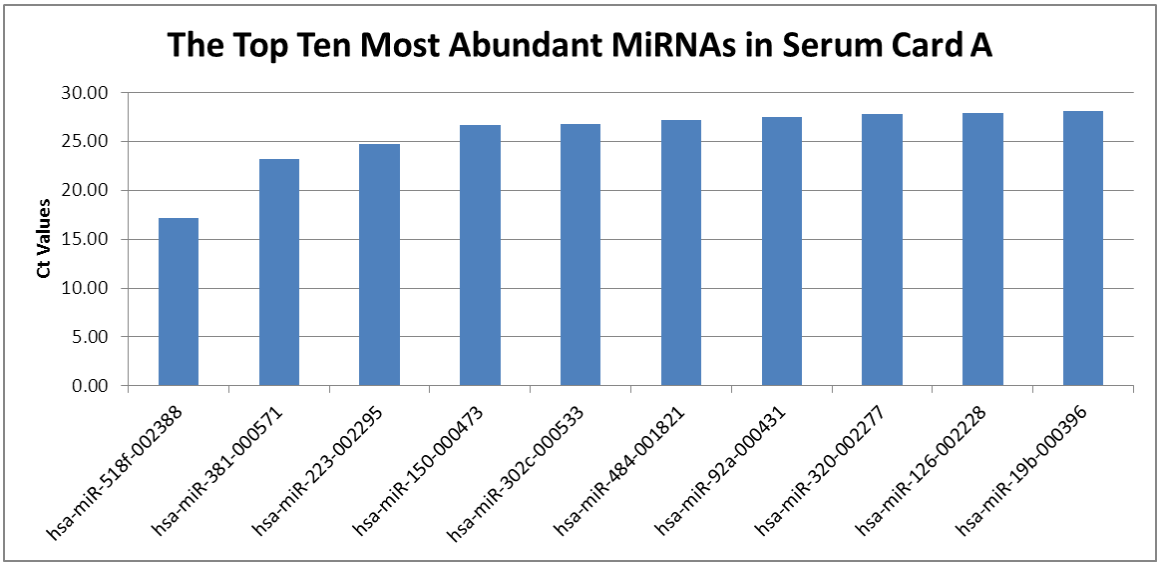
MiRNA screening (Megaplex)

To identify miRNA which may be differentially expressed in serum and urine in the context of proteinuric kidney disease, I screened one serum sample from a normal donor and two serum samples from patients with kidney disease and two urine samples from normal donors and two urine samples from patients with kidney disease using the Taqman Array miRNA cards. Each Taqman Array MiRNA Card was embedded with 377 miRNA targets. When comparing normal and disease samples for serum Card A, 101 out of a total of 377 miRNA targets had fully detectable signals ($Ct < 35$) in at least one of the three samples. When comparing normal and disease samples for urine Card A and Card B, 146 out of 377 and 61 out of 377 miRNA targets, respectively, showed fully detectable signals in at least two of the four samples.

The top ten most abundant miRNAs in each biofluid are summarized as follows (Figures 1, 2 and 3). These three lists were generated based on the ranks of the average Ct values of all screening samples in each matrix. A smaller Ct value represents higher expression level as they are reversely correlated. The lists generated in this study were compared to the list created by Weber *et al*, who conducted a study to evaluate the miRNA spectrum in 12 body fluids including plasma and urine; however, the results for the most abundant miRNAs in plasma and urine were surprisingly unmatched with no overlap in the serum lists and only miR-223 shared between the urine lists. The differences suggest that the lists generated by Weber *et al* may not represent abundance across all individual and all studies.

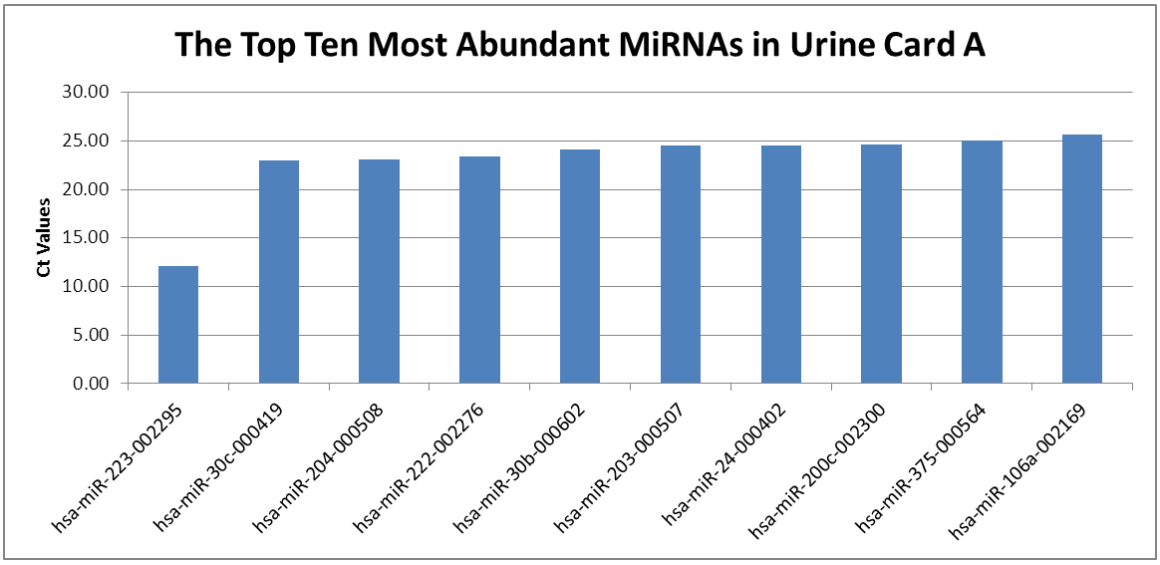
For both serum and urine matrices, a number of specific miRNA targets were selected based on the miRNA screening results. The selected miRNA targets were the ones that were 1) robustly amplified in the screening assay, and 2) displayed the most significant difference in expression level between normal and disease samples. These individual targets were further evaluated by singleplex miRNA Taqman Assay, which examined miRNA expression between normal and disease groups. For the serum analysis, seven miRNA targets showed a ΔΔCt between normal and disease samples of greater than six (Table 7). I selected the top three miRNA targets from the screening results – hsa-miR-93, hsa-miR-627, and hsa-miR-146b for further analysis with target specific Taqman Assays. For the urine analysis, a total of eight miRNA targets had ΔΔCt values greater than five (Tables 8 & 9) and I selected the top four miRNA targets from the screening results – hsa-miR-451, hsa-miR-629, hsa-miR-642, and hsa-miR-1244 for further analysis using Taqman Assays. In each target specific Taqman Assay, cel-miR-39, hsa-miR-16 and hsa-miR-223 were included as normalizing controls. In addition, hsa-miR-21 was evaluated from both serum and urine as its level in kidney tissue was reported to be positively associated with kidney fibrosis in mice (Chau et al., 2012).

Figure 1: The Top Ten Most Abundant MiRNAs in Serum Card A



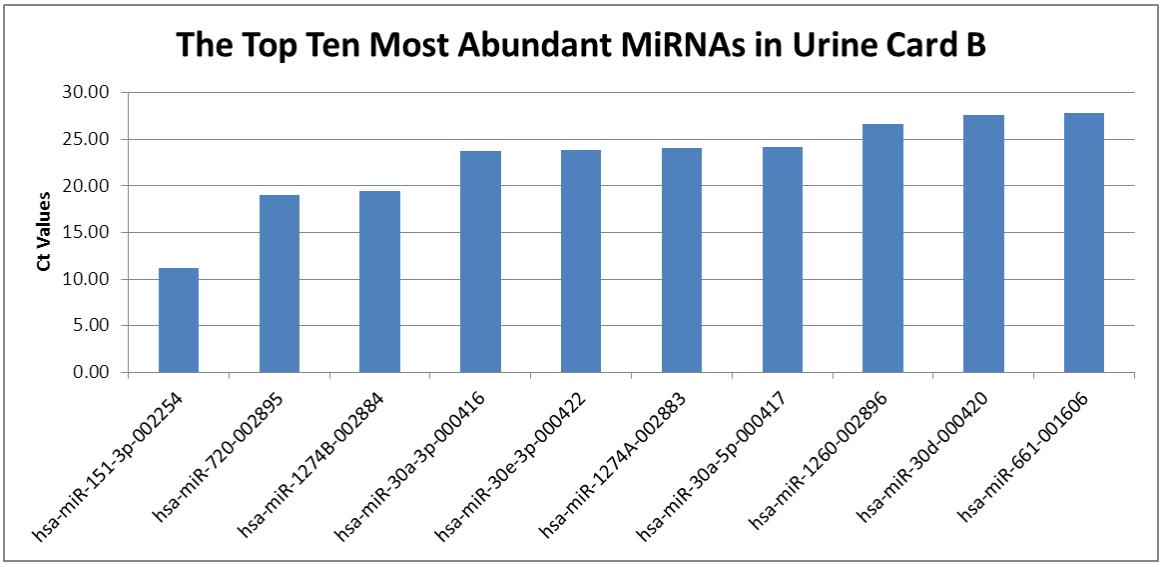
The Ct values are reversely proportional to the expression level. The miRNA species are arranged in descending order with the highest concentration on the left.

Figure 2: The Top Ten Most Abundant MiRNAs in Urine Card A



The Ct values are reversely proportional to the expression level. The miRNA species are arranged in descending order with the highest concentration on the left.

Figure 3: The Top Ten Most Abundant MiRNAs in Urine Card B



The Ct values are reversely proportional to the expression level. The miRNA species are arranged in descending order with the highest concentration on the left.

Table 7: Serum Card A screening results.

No.	Sample	Detector	Ct	ΔCt	Avg ΔCt	ΔΔCt
1	Normal serum	hsa-miR-122-002245	40.00	8.28	8.28	6.50
	Disease serum 1		33.10	1.78	1.78	
	Disease serum 2		32.71	1.79		
2	Normal serum	hsa-miR-20b-001014	40.00	8.28	8.28	7.72
	Disease serum 1		31.90	0.58	0.56	
	Disease serum 2		31.47	0.55		
3	Normal serum	hsa-miR-27a-000408	40.00	8.28	8.28	6.73
	Disease serum 1		33.13	1.81	1.56	
	Disease serum 2		32.22	1.30		
4	Normal serum	hsa-miR-411-001610	32.83	1.11	1.11	-7.77
	Disease serum 1		40.00	8.68	8.88	
	Disease serum 2		40.00	9.08		
5	Normal serum	hsa-miR-454-002323	40.00	8.28	8.28	6.29
	Disease serum 1		34.98	3.66	1.99	
	Disease serum 2		31.25	0.33		
6	Normal serum	hsa-miR-627-001560	23.67	-8.05	-8.05	-10.58
	Disease serum 1		27.31	-4.02	2.53	
	Disease serum 2		40.00	9.08		
7	Normal serum	mmu-miR-93-001090	40.00	8.28	8.28	8.22
	Disease serum 1		32.20	0.88	0.06	
	Disease serum 2		30.17	-0.75		
8	Normal serum	hsa-miR-146b-001097	31.87	0.15	0.15	-2.93
	Disease serum 1		34.26	2.94	3.08	
	Disease serum 2		34.14	3.22		
9	Normal serum	hsa-miR-21-000397	30.89	-0.83	-0.83	-0.78
	Disease serum 1		32.52	1.20	-0.04	
	Disease serum 2		29.64	-1.28		

The four miRNAs (No.6-9) highlighted in red were further evaluated using target specific Taqman Assays.

Table 8: Urine Card A screening results.

No.	Sample	Detector	Ct	ΔCt	Avg ΔCt	ΔΔCt
1	Normal urine 1	hsa-miR-486-001278	40.00	9.89	10.33	5.71
	Normal urine 2		40.00	10.77		
	Disease urine 1		32.51	3.39	4.62	
	Disease urine 2		36.28	5.84		
2	Normal urine 1	hsa-miR-629-002436	40.00	9.89	10.33	6.68
	Normal urine 2		40.00	10.77		
	Disease urine 1		33.16	4.04	3.65	
	Disease urine 2		33.70	3.26		
3	Normal urine 1	hsa-miR-642-001592	33.07	2.96	3.18	-7.04
	Normal urine 2		32.64	3.41		
	Disease urine 1		40.00	10.88	10.22	
	Disease urine 2		40.00	9.56		
4	Normal urine 1	hsa-miR-874-002268	33.53	3.42	3.46	-5.35
	Normal urine 2		32.73	3.50		
	Disease urine 1		40.00	10.88	8.81	
	Disease urine 2		37.17	6.73		
5	Normal urine 1	mmu-miR-451-001141	40.00	9.89	8.89	9.48
	Normal urine 2		37.13	7.90		
	Disease urine 1		27.10	-2.02	-0.59	
	Disease urine 2		31.29	0.84		
6	Normal urine 1	hsa-miR-21-000397	27.343	-2.767	-2.776	0.6975
	Normal urine 2		26.445	-2.785		
	Disease urine 1		24.484	-4.636	-3.4735	
	Disease urine 2		28.129	-2.311		

The four targets highlighted in red were further evaluated using target specific Taqman Assays.

Table 9: Urine Card B screening results.

No.	Sample	Detector	Ct	ΔCt	Avg ΔCt	ΔΔCt
1	Normal urine 1	hsa-miR-1227-002769	34.79	3.67	3.62	-5.56
	Normal urine 2		34.30	3.57		
	Disease urine 1		40.00	9.06	9.18	
	Disease urine 2		40.00	9.29		
2	Normal urine 1	hsa-miR-1244-002791	33.82	2.70	1.72	-7.45
	Normal urine 2		31.48	0.75		
	Disease urine 1		40.00	9.06	9.18	
	Disease urine 2		40.00	9.29		
3	Normal urine 1	hsa-miR-320B-002844	34.37	3.25	2.90	-6.28
	Normal urine 2		33.27	2.54		
	Disease urine 1		40.00	9.06	9.18	
	Disease urine 2		40.00	9.29		

MiR-1244 highlighted in red was further evaluated using target specific Taqman Assays.

MiRNA Taqman Assay (Singleplex)

To evaluate the top targets from each matrix, I examined the levels of two of the serum targets, hsa-miR-93 and hsa-miR-627, along with miR-21 and miR-146b which have been reported to increase in the kidneys of mouse models with kidney fibrosis and chronic renal inflammation, respectively, and four of the urine targets, miR-451, miR-629, miR-642 and miR-1244, together with miR-21in a larger sample set using singleplex Taqman Assays. Overall, ΔΔCt's obtained from individual Taqman Assays were much less significant than from the Taqman Array miRNA Cards. On the serum side, out of the four targets analyzed, three (miR-93, miR-16 and miR-223) showed the same expression trends as the screening result (Table 10 and Figure 4); targets elevated in the disease samples from the megaplex screening assay also showed an elevation in disease with the singleplex Taqman Assay. In contrast, the expression profile for miR-627 flipped; this may be due to its extremely low expression that was very close to the limit of detection (Ct>35). The target with the most significant ΔΔCt was miR-146b, which decreased in all six disease samples as compared to the five normal samples with an average difference of 1.46 cycles (2.75 fold; p=0.000 unpaired t-test). The expression level of miR-21 was also significantly different between the normal and disease sample groups with a p=0.003 (unpaired t-test). On the urine side, all five miRNA targets (miR-21, miR-451, miR-629, miR-642 and miR-1244) exhibited the same expression profile as the screening result, although the expression differences between normal and disease samples were much smaller (Table 11 and Figure 5). MiR-21 showed the biggest difference in expression between the six normal urine samples and three disease urine samples with ΔΔCt of 1.30,

indicating that on average miR-21 was expressed nearly 2.46 fold higher in the disease group; however, these differences were not significant ($p=0.23$). MiR-451 was expressed on average 1.16 cycles (nearly 2.23 fold) higher in disease group as compared to normal group but again the difference was not significant ($p>0.05$). Expression profiles of the miRNA targets in serum and urine are shown in Figures 4 and 5. The expression levels of the normal groups are set to one and the Ct values are converted to expression levels using the formula (expression level = 2^(Ct)) for easier comparison.

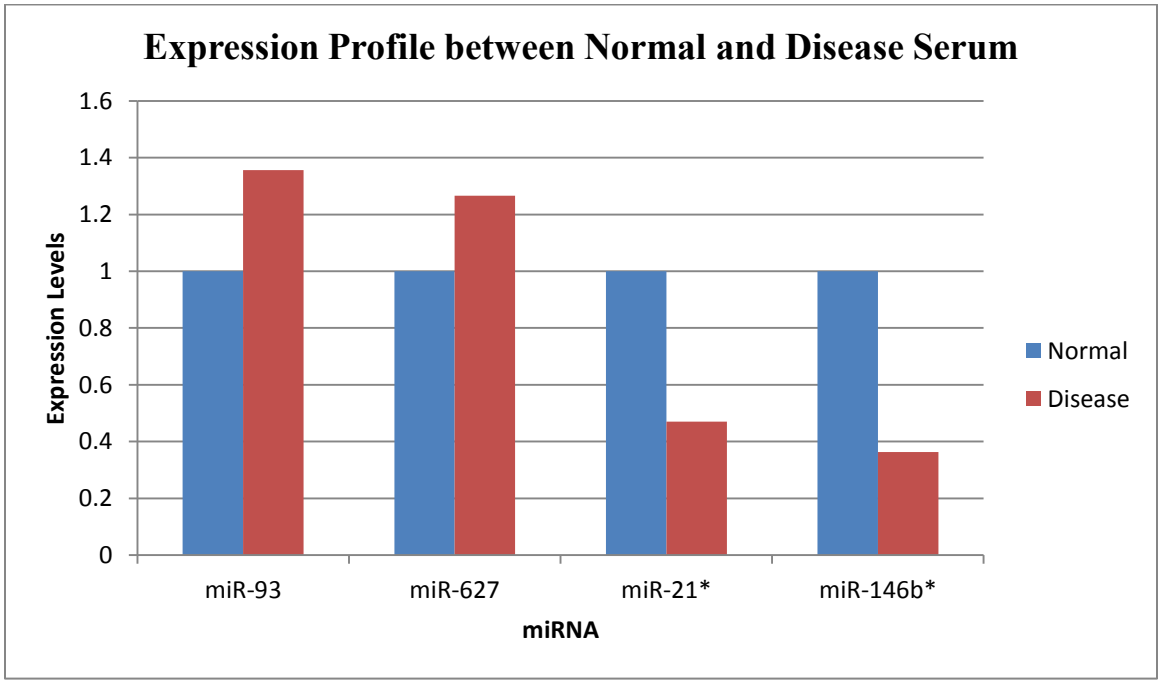
Table 10: Summary of normal vs. disease serum expression results.

miRNA Target	Sample	Ct	ΔCt	Ave ΔCt	ΔΔCt
hsa-miR-93	Normal1	34.94	8.19	8.27	0.44
	Normal2	35.35	9.12		
	Normal3	33.06	8.22		
	Normal4	31.27	7.15		
	Normal5	33.91	8.85		
	Normal6	32.82	8.09		
	Disease1	33.82	9.22	7.83	
	Disease2	31.94	8.33		
	Disease3	33.25	7.13		
	Disease4	32.29	7.76		
	Disease5	33.29	6.92		
	Disease6	31.77	7.59		
hsa-miR-627	Normal1	40.00	13.25	14.61	0.34
	Normal2	40.00	13.77		
	Normal3	40.00	15.16		
	Normal4	40.00	15.89		
	Normal5	39.38	14.32		
	Normal6	40.00	15.26		
	Disease1	39.15	14.55	14.27	
	Disease2	38.45	14.85		
	Disease3	39.71	13.58		
	Disease4	37.69	13.16		
	Disease5	40.00	13.64		
	Disease6	40.00	15.82		
hsa-miR-21	Normal1	26.11	5.79	5.93	-1.10
	Normal2	25.68	5.32		
	Normal3	25.70	5.94		
	Normal4	26.31	6.47		
	Normal5	25.64	6.12		
	Disease1	29.92	7.25	7.02	
	Disease2	28.41	7.04		
	Disease3	28.84	6.87		
	Disease4	29.94	7.08		
	Disease5	27.25	7.22		
	Disease6	28.36	6.67		
hsa-miR-146b	Normal1	27.67	7.35	7.51	-1.46
	Normal2	27.56	7.20		
	Normal3	27.41	7.65		
	Normal4	27.84	8.00		
	Normal5	26.88	7.36		
	Disease1	32.45	9.78	8.97	
	Disease2	30.25	8.88		
	Disease3	30.60	8.63		
	Disease4	31.66	8.80		
	Disease5	29.28	9.26		
	Disease6	30.15	8.46		

Table 11: Summary of normal vs. disease urine expression results.

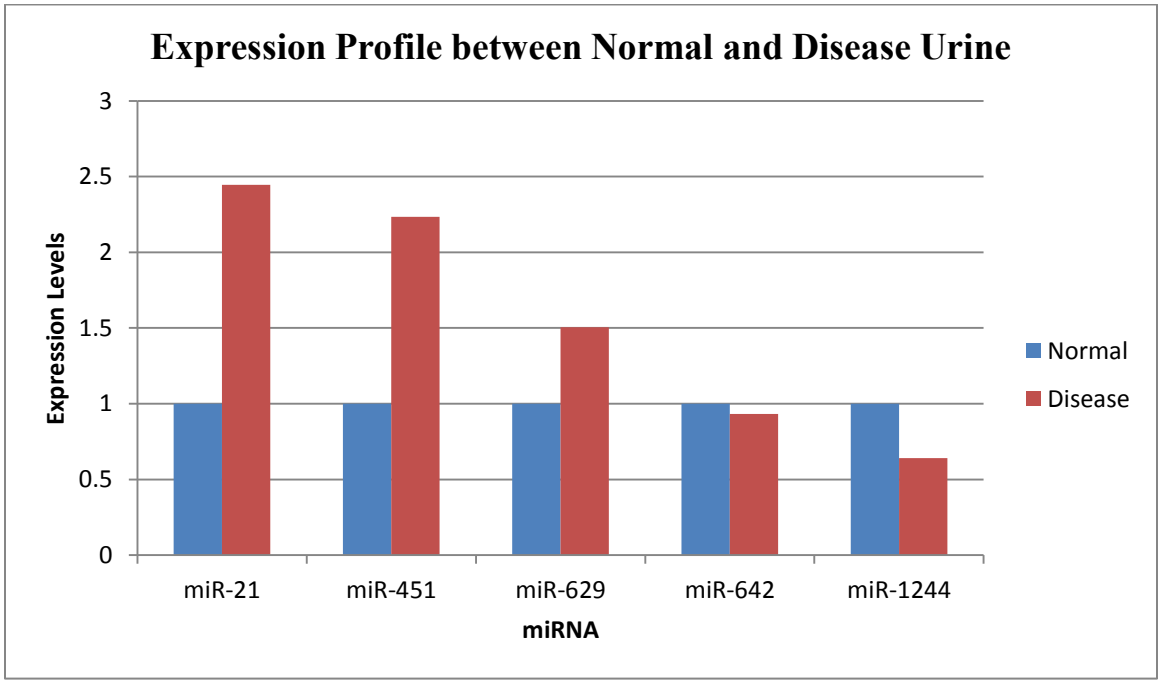
miRNA Target	Sample	Ct	ΔCt	Ave ΔCt	ΔΔCt
hsa-miR-21	Normal1	28.62	3.81	3.67	1.30
	Normal2	30.82	3.79		
	Normal3	32.03	2.75		
	Normal4	32.64	4.62		
	Normal5	29.14	3.84		
	Normal6	22.90	3.23		
	Disease1	31.03	3.36	2.38	
	Disease2	28.43	0.98		
	Disease3	26.87	2.79		
hsa-miR-451	Normal1	31.30	6.49	5.67	1.16
	Normal2	31.19	4.16		
	Normal3	31.66	2.38		
	Normal4	31.58	3.56		
	Normal5	32.08	6.78		
	Normal6	30.33	10.66		
	Disease1	32.09	4.42	4.51	
	Disease2	31.74	4.29		
	Disease3	28.90	4.82		
hsa-miR-629	Normal1	35.53	10.72	9.29	0.59
	Normal2	34.81	7.78		
	Normal3	35.62	6.34		
	Normal4	35.72	7.70		
	Normal5	35.84	10.54		
	Normal6	32.31	12.64		
	Disease1	36.20	8.53	8.70	
	Disease2	35.42	7.98		
	Disease3	33.66	9.58		
hsa-miR-642	Normal1	36.44	11.63	12.72	-0.09
	Normal2	40.00	12.97		
	Normal3	40.00	10.72		
	Normal4	40.00	11.98		
	Normal5	40.00	14.71		
	Normal6	34.00	14.33		
	Disease1	38.41	10.74	12.82	
	Disease2	39.24	11.79		
	Disease3	40.00	15.92		
hsa-miR-1244	Normal1	36.76	11.95	11.95	-0.64
	Normal2	40.00	12.97		
	Normal3	40.00	10.72		
	Normal4	40.00	11.98		
	Normal5	35.02	9.73		
	Normal6	34.02	14.35		
	Disease1	40.00	12.33	12.59	
	Disease2	40.00	12.55		
	Disease3	36.96	12.88		

Figure 4: Comparison of Expression Profile between Normal and Disease Serum.



*The reduced expression of miR-21 and miR-146b in disease samples is statistically significant with $p<0.05$.

Figure 5: Comparison of Expression Profile between Normal and Disease Urine.



Chapter IV

Discussion

In this study, I established robust extraction and amplification procedures to measure circulating miRNA level in serum and urine that can be used for the identification and evaluation of potential disease biomarkers. Using these procedures, I identified a list of miRNA targets that were differentially expressed in serum and urine between healthy normal donors and patients with proteinuria or nephrotic syndromes. A subset of miRNA targets with the greatest alteration in expression level were further evaluated using individual Taqman Assays. For the serum analysis, miR-21 and miR-146b were significantly down-regulated in the disease group. For the urine analysis, miR-21 and hsa-miR-451 showed elevated expression levels in nephrotic urine as compared to normal controls; however, the differences were not statistically significant.

Method Optimization

For RNA isolation, Trizol-LS provides an inexpensive and reliable method for the extraction of miRNA from both serum and urine. The inclusion of glycogen as a carrier resolved isolation challenges related to the low starting concentration of circulating RNA in both serum and urine. Further optimization of other extraction parameters, including sample collection tubes and starting sample volumes, resulted in a final protocol that, in

conjunction with the ABI RT and PCR, offers high intra-assay precision with %CV values of less than 2.55 and 2.37 in serum and urine, respectively. While column-based commercial kits are reported to be user friendly, the RNA yield from these kits could be variable between kit lots (Bentis et al., submitted) and such variability may add another layer of uncertainty to miRNA quantitation.

The extraction and qPCR protocols used here were effective in detecting miRNA in human serum and urine samples. Both extraction and PCR precision studies showed very small %CV and maximal Ct difference when the same assay was repeated for up to eight times, indicating the reproducibility of this method. Results from the precision study suggested that any Ct difference greater than 2.18 was likely to be true expression difference not caused by intra-assay variation. The freeze-thaw stability study demonstrated consistent assay results for serum samples that had gone through multiple freeze-thaw cycles. The same analysis was not performed for the urine samples but literature has shown high stability and quality of miRNA in urine and saliva (Blondal et al., 2012). Freeze-thaw stability is particularly important because most of the disease samples we received from Bioreclamation were collected in the past and had been stored in their inventory where freeze-thaw information was not available. The results of this study indicate that miRNA in serum is stable and that samples with variable freeze thaw numbers can be compared without confounding the results.

Normalization

A robust normalization tool is critical for miRNA expression analysis due to the inability to quantify RNA concentration prior to analysis. A significant challenge for miRNA screening using the Taqman Array MiRNA Card was the lack of a consensus normalization method. The U6 endogenous control embedded in each card was not ideal in our case because U6 is not consistently expressed in human biofluids such as serum and urine. In a recent publication, Wylie and his colleagues compared seven normalization methods and proposed a novel mean-centering normalization method that performs the best among all candidates (Wylie, 2011). The mean-centering method used the average Ct value of all fully detected miRNA targets in each card as the normalizer (Ct < 35 was considered as fully detected). By adopting Wylie's mean-centering normalization method, I was able to better evaluate all miRNA targets across samples and select the ones with the largest differences in gene expression.

Although mean-centering normalization method works well in the megaplex Taqman Array Card platform, it is not suitable in singleplex miRNA Taqman Assays which only examine one target at a time. The normalization strategy for singleplex Taqman Assays aims to identify normalizer miRNAs whose expression values across samples most closely track the mean value of all miRNAs. In this study, I employed the normalization method from Kroh *et al.* by using miR-16 and miR-223, as both are expressed at high levels in plasma and serum and are relatively invariant across large numbers of samples (Kroh, Parkin, Mitchell, & Tewari, 2010). Furthermore, miR-223 was shown to be among the most abundant miRNA in urine (Weber et al., 2010)

Top Abundant miRNAs

Identifying robust and invariantly expressed miRNA targets in various body fluids is critical not only because it provides an overview of the expression pattern, but it also helps to uncover additional internal miRNA controls in each sample matrix for future studies. The screening assays using Taqman Array MiRNA Cards A and B resulted in a list of the most abundant miRNAs in the serum and urine samples evaluated in the study (Figures 1, 2 and 3). Comparison of the results from this study with published miRNA lists from Weber and colleagues show minimal overlap. Weber *et al* evaluated the miRNA spectrum in 12 body fluids from five healthy donors and generated a list of top twenty most abundant miRNAs in each fluid type (Weber et al., 2010). Most miRNA targets from Weber's top twenty list can also be found in this screening study but at lower expression levels. MiR-223, however, is common to both urine lists and was used as a normalizer in this study for the single target urine Taqman assays. The mismatch may be explained by procedural differences; Weber *et al* used Qiagen kits for the RNA extraction and qPCR arrays on samples also collected from Bioreclamation. Ideally miRNA abundance should not vary across technical platforms. The possibility suggested by this data indicates that extraction and amplification techniques may bias miRNA target detection limiting the ability to compare results across studies and confounding the identification of significant biomarkers. As this was a very limited study, further work to compare various procedures and commercial kits may help to understand variability they introduce into the detection process.

The most abundant miRNAs in urine (Figure 2) include miR-30c and miR-24; the two miRNAs were reported to be robustly expressed in adult kidney and their expression was confirmed by Northern blot (Ho et al., 2008). The positive correlation of these two miRNAs between urine and kidney suggests their potential role as a non-invasive disease indicator. Nephrotic syndromes such as FSGS with depleted podocytes may result in abnormal shedding of certain kidney miRNAs. In my screening result, miR-24 was elevated in the two urine samples from patients with proteinuria but the difference was not significant due to small sample size ($\Delta\Delta Ct=0.76$, $p>0.05$).

Target Identification

A total of 377 and 754 miRNA targets were screened for in serum and urine, respectively. For serum analysis, 101 out of a total of 377 targets had fully detectable signals ($Ct < 35$) in at least one of the three samples. For urine analysis, 207 out of 754 targets showed fully detectable signals in at least two of the four samples. Among these fully detected targets, I further reduced the target number by eliminating the ones with less than three cycle difference between normal and disease groups. This resulted in 27 candidates in serum and 32 in urine. Although the significance of the results was impacted by the small sample size, the screening result pointed to targets meriting deeper analysis using singplex Taqman Assay.

On serum card A, the expression of miR-146b was significantly lower in the disease group as compared to the normal group. This result is in contrast to data from a recent publication by Ichii and his colleagues who observed elevated levels of miR-

146a/b with the development of chronic renal inflammation in B6.MRLc1 CKD mouse kidneys (Ichii et al., 2012). The discrepancy between Ichii's and my data may be caused by the sample type (kidney biopsy vs. serum or urine) and sample origin (mice vs. human). Some studies suggest that miRNA expression in tissue maybe inversely associated with its surrounding biofluid (Waters et al., 2012). Leakage of miRNA into the surroundings may result in lower expression within the tissue itself and higher expression in circulating blood. MiR-93 was another interesting target as it was noted in a review paper by Bhatt and his colleague that miR-93 expression was decreased in kidney microvascular endothelial cells and glomeruli of diabetic *db/db* mice (Bhatt, Mi, & Dong, 2011). In the study described here, miR-93 was slightly up-regulated in disease serum as compared to normal serum, also indicating a possible inverse association between levels in the kidney and surrounding blood in certain conditions.

In the urine matrix, none of the five miRNA targets evaluated using the singleplex miRNA Taqman Assays showed significant differences between the normal and disease samples. The failure to detect significant differences is likely tied to the limited sample size used for screening and verification. Although not statistically significant, two miRNAs, miRNA-21 and miR-451 trended towards higher levels in the disease samples. Noteworthy, the results for miR-21 consistently aligned with findings from another published study, which detected elevated levels of miR-21 in mice with kidney injury/fibrosis (Chau et al., 2012). Chau's work demonstrated that miR-21contributes to fibrogenesis and epithelial injury in the kidney in two mouse models and suggests miR-21 as a candidate target for antifibrotic therapies. The results here support the association

of miR-21 levels and disease status, although a larger sample set is needed for further validation.

Overall, the results from the individual Taqman Assays did not recapitulate the fold change differences observed in the screening assay. The discrepancy between the megaplex and the singleplex formats may result from differences in the assay procedures. The 384-well megaplex platform utilized a pre-amplification step which was not included in the singleplex assays. The different normalization methods used in the screening and individual Taqman Assays may also contribute to the discrepancy noticed between the two platforms. In comparable studies published elsewhere, several different screening platforms from other vendors were utilized and thus the degree of correlation between singlex and megaplex platforms is unclear. Further studies with a larger sample set and universal normalization method is required to better characterize the data correlation between Taqman MiRNA Arrays (multiplex platform) and Taqman Assays (singplex platform).

One of the greatest challenges in this study was sample size and condition. Limited access to patient samples with specific disease types undermined the power of the study. As shown in Table 2, the patient samples used in this study all present with a complicated disease spectrum. In addition to CDK and/or nephrotic syndrome, the patients selected for the serum analysis all also have type II diabetes which confounds the detection of biomarkers that may be specific for kidney disease as targets may correlate with either phenotype. Because kidney disease may arise from a diverse set of etiologies, initial identification studies may benefit from use of model organisms with very defined pathologies and limited comorbidities. This is suggested by the analysis of miR-21, a

target that was initially identified in mouse models and later verified in the patients. In addition, larger studies looking at well-defined patient populations (for example patients with FSGS) or patients with CDK and nephrotic with a variety of causes will be helpful in clarifying potential kidney disease biomarkers. Moreover, disease samples in use here were mostly inventoried specimens that had been stored at $\leq$-60^{o}C for over a year, while normal samples were freshly frozen. The age and ethnicity of the samples may also play a role as most disease samples came from older Caucasian patients whereas the normal samples were collected from younger Black and Hispanic donors. The small sample size of less than ten specimens for serum and urine, made it more difficult to draw statistical conclusions. Despite these limitations, data support other published findings suggesting that candidate miRNAs identified here are worth further investigation in a larger sample set.

Conclusion

The extraction and qPCR method developed herein has proved to be effective and reproducible using standard lab equipment and reagents. Circulating miRNAs present in serum are stable up to at least five freeze-thaw cycles. The screening assay suggests a list of miRNA targets that are differentially expressed in the serum and urine of patients with chronic kidney disease. The expression trend obtained from the screening was verified by individual Taqman Assays, although the magnitude of difference was smaller in the singleplex platform. For serum analysis, both miR-21 and miR-146b appear to be significantly down-regulated in disease serum. On the urine side, miR-21 was slightly up-

regulated in nephrotic urine but the difference fails to reach statistical significance. A larger sample set with more specific disease status is required to further evaluate the miRNA targets uncovered in this study. The screening results also suggest a list of most abundant miRNAs in the two fluid types which have the potential to serve as internal controls for future studies. The Trizol-LS extraction method combined with Taqman qPCR is easily expandable to other matrices and targets such as exosomes and mRNAs (messenger RNAs). In patients with severe proteinuria such as FSGS, damaged podocytes containing a variety of miRNAs are depleted into urine in the form of exosomes. It will be interesting to examine miRNA levels in urinary exosomes as the next step in follow-up studies.

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