Diagnosis of Interstitial Fibrosis and Tubular Atrophy in Kidney Allograft Implementation of MicroRNAs

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INTRODUCTION

Chronic allograft nephropathy is the major cause of kidney allograft loss, and recent advances in immunosuppression therapy do not alter the picture. Chronic allograft nephropathy is a complex phenomenon that manifests with a progressive decline in glomerular filtration rate, and histologically, it is characterized by a progressive interstitial fibrosis and tubular atrophy (IFTA). Interstitial fibrosis and tubular atrophy is an early event that starts early after engraftment and even could be found in recipients with good allograft function.1 It is reported that almost all allografts finally develop chronic allograft nephropathy, and IFTA starts as early as the first year of transplantation.2,3 The incidence of IFTA has been reported to be 50%, 70%, and 100% at the 1st, 2nd, and 10th years after transplantation, respectively.4,5 Renal biopsy is the current gold standard for the exact evaluation of IFTA.6,8 Serum creatinine and estimated glomerular filtration rate have limited clinical roles in estimating the histopathological changes and graft fibrosis.6,9 Renal biopsy is an invasive procedure. It requires hospitalization, and sampling errors bias and inter-observer variability all remain the clinical challenges of biopsy.10,11 Therefore, noninvasive, sensitive, and etiology-specific biomarkers are critically needed. The most recent discoveries of genetic biomarkers have arisen in the field of microRNAs. Analysis of gene expression at microRNA and protein levels has been reported as a predictor of renal fibrosis.1,12-23 MicroRNAs are endogenous single-stranded RNA, and up until now, over 24,521 microRNAs are reported (http://www.mirbase.org). MicroRNAs regulate gene expression by translation inhibition or induction of microRNAs degradation, and therefore, modulate diverse biological processes. MicroRNAs are implicated in cellular proliferation, differentiation, apoptosis, organ development, stem cell biology, tumor genesis, and metastasis, as well as functional regulation of the immune system.24,25 Distinct sets of microRNAs are found in different cell types and tissues, and their aberrant expression is associated with numerous human diseases, and microRNAs are emerging not only as potential biomarkers, but also as potential therapeutic targets in different diseases.26-31 In the scenario of renal fibrosis, microRNAs play a conductor role in transforming growth factor-ß (TGF-ß) signaling, extracellular matrix (ECM) accumulation, and epithelial-mesenchymal transformation (EMT).
INTERSTITIAL FIBROSIS AND TUBULAR ATROPHY

Mechanisms and Mediators

The mechanisms that lead to IFTA are multifactorial, including both immunological and nonimmunological factors. Interstitial fibrosis and tubular atrophy arises from an orchestrated deregulation of epithelial cells, fibroblasts, myofibroblasts, fibrocytes, endothelial cells, lymphocytes, monocyte and macrophages, and their secreted cytokines. Among those cytokines, TGF-β1 has a key role. Transforming growth factor-β1 signaling pathway leads to proliferation of fibroblasts and myofibroblasts, EMT, and excessive ECM accumulation (Figure 1). The activated fibroblasts or myofibroblasts are either residential fibroblasts or derived from vascular pericytes. Tubular atrophy that is defined by a decrease in tubular diameter and number is one of the characteristic features of IFTA.
MICRONRMA AND RENAL FIBROSIS
Transforming Growth Factor-β1-induced Fibrosis

Recent discoveries suggest that kidney microRNAs differ from other organs' microRNAs. Normally, they have regulatory roles in kidney development and function, and they could be a valuable tool for understanding, diagnosing, and treatment of different renal diseases. The TGF-β/Smad3 pathways play a major role in tissue fibrosis. During renal injury, TGF-β signaling is upregulated and stimulates TGF-β1 receptor that then activates Smad3 pathway. In the context of renal fibrosis, Smad3 is pathogenic, whereas Smad7 is protective.

MiR-433 is an important component of TGF-β/Smad3 pathways and imposes a positive feedback loop and amplifies TGF-β/Smad3 signaling. In vitro and in vivo expression of miR-433 enhances TGF-β1-induced fibrosis by enhancing the antizyme inhibitor, Azin1, that is an important regulator of polyamine synthesis.

MicroRNAs in Regulating Epithelial-Mesenchymal Transformation

Tubular EMT is of the major mechanisms involved in renal fibrosis. Epithelial-mesenchymal transformation is a highly regulated pathological event in which epithelial cells finally lose their adhesion and apical and basal polarity, and transform to migratory, spindle-shaped, elongated mesenchymal cells. Transforming growth factor-β signaling is a potent inducer of EMT through activation of mesenchymal transcription factors that are zinc finger E-box-binding homeobox proteins 1 and 2 (ZEB1/2). About 35% of the fibroblasts that are central for the development of progressive renal fibrosis are derived from EMT. Several microRNAs, particularly, miR-192, miR-200, and miR-30 families, play a major role in the induction of EMT in renal tubular cells. One potential driver of EMT and IFTA is chronic hypoxia that induces increase in matrix metalloproteinase-2 expression via reduction of miR-124.

MiR-192. In vitro and in vivo studies have suggested that miR-192 mediates the development of tubulointerstitial fibrosis via repression of ZEB1/2. MiR-192 upregulation, downregulates the ZEB2 and increases the expression of collagen 1 and 2. In another study, overexpression of miR192 and deletion of Smad7 promoted fibrosis in obstructive kidney disease. Upregulation of miR-192 is also reported in IgA nephropathy and hypertensive nephrosclerosis. Locked nucleic acid inhibitor of miR-192 decreases renal miR192 levels and reduces renal hypertrophy and fibrosis in diabetic mice. MiR-192-knockout mice are also protected from diabetic nephropathy. Very recently, Hong and colleagues have found that vascular endothelial growth factor, a renal tubular epithelial survival factor, can suppress Smad3 and miR-192, and subsequently inhibits EMT induction by TGF-β1 in human kidney cortex cell line. In contrast to those findings, Krupa and colleagues claimed that TGF-β1 induction could suppress miR-192 expression in human tubular epithelial cells. They also observed that loss of miR-192 expression in mice with diabetic nephropathy was associated with increased fibrosis through downregulation of E-cadherin. A recent study of Glowacki and associates indicates reduction of miR-192 in the serum of kidney allograft recipients. Taken together, miR-192 exhibits both pro- and anti-fibrotic properties depending on the cell phenotype. Several E-boxes were found in the upstream promoter regions of Col1a2, Col4a1, miR-216a/217, and the miR-200 family. Downregulation of E-box repressors such as ZEB1/2 by miR-192 resulted in increased expression of miR-216a and miR-217. They increase collagen production through downregulation of phosphatase and tensin homolog and activation of akt kinase signaling to promote hypertrophy in cultured murine mesangial cells. Increased levels of miR-216a and miR-217 in turn led to the upregulation of TGF-β. Fiorentino and coworkers also found that increased expression of miR-217 targeted tissue inhibitor of metalloproteinase-3 through downregulation of SirT1.

MiR-200 family. MiR-200 family encompasses miR-200a, miR-200b, miR-200c, miR-141, and miR-429. This family of microRNAs are acting through suppression of the posttranscriptional expression of ZEB1/2. They prevent TGF-β-mediated EMT through suppression of ZEB1/2 and TGF-β2. Tang and colleagues indicated that microRNA-200b suppresses TGF-β1-induced EMT via inhibition of ZEB1/2 and fibronectin by direct targeting of their 3’ untranslated region mRNA.
obstruction mice models of kidney fibrosis, miR-200 family had controversial results. Wang and associates reported that in both early and advanced mice model of diabetic nephropathy proximal tubular epithelial cells, miR-200a was downregulated. Obvious downregulation of miR-200a, miR-200b and miR-141 in unilateral ureter obstruction kidneys have also been reported in other studies. Elevated intrarenal expression of miR-200a, miR-200b, miR-141, miR-192, miR-205, and miR-429 were found in renal biopsies of patients with hypertensive glomerulosclerosis, and the degree of upregulation was correlated with severity of disease. It has been demonstrated that levels of miR-200b/c increase in response to TGF-β stimulation and after introduction of miR-192 in mouse mesangial cells. In the same study, inhibitors of miR-192 reduced the expression of miR-200b/c, Col1a2, Col4a1, and TGF-β1. Downregulation of the miR-200 family, especially mir-200b, initiates the dedifferentiation of renal tubular cells and progression of renal fibrosis, and it would be an important target for novel therapeutic strategies.

**MiR-30.** MiR-30 family is abundantly expressed in the kidney and comprises miR-30a to miR-30e. They have similar seed sequence in their 5’ terminus. MiR-30 family is required for pronephron’s development and podocyte homeostasis. During in vitro and in vivo renal fibrosis process, the expression of miR-30e is markedly downregulated, and miR-30e directly inhibits TGF-β1-induced EMT by targeting mitochondrial uncoupling protein-2 mRNA. Low levels of miR-30b and miR-30c expression is associated with kidney fibrosis. Targeting the above pathway may have some therapeutic implications for halting the kidney fibrosis.

**MicroRNAs Regulation on Extracellular Matrix Proteins**

**MiR-29 families.** The best examples of microRNAs’ regulation on extracellular matrix proteins are miR-21 and miR-29 families. The human miR-29 family comprises of 4 members: miR-29a, miR-29b1, miR-29b2, and miR-29c, all of which targeting the same genes. The miR-29 family targets a large number of ECM genes including collagen types 1A1, 3A1, 4A1, 5A1, 5A2, 5A3, 7A1, and 8A1 and fibrillin and act as anti-fibrotic microRNAs. MiR-29 family functions as a downstream inhibitor of TGF-β/Smad3-pathways-mediated fibrosis. This family also acts as an inhibitor of TGF-β-mediated deposition and remodeling of ECM. Conversely, in cultured human proximal tubular epithelial cells, high glucose media and TGF-β stimulation downregulate miR-29a and contributes to expression of multiple collagen genes.

Expression of miR-29 is decreased in urine samples of patients with IgAnephropathy. Long and colleagues reported that miR-29c is an important regulator of hyperglycemia-induced apoptosis of podocytes, and in vivo knockdown of miR-29 leads to a decrease in glomerular apoptosis, fibronectin expression, and glomerular ECM accumulation. Downregulation of miR-29c has been reported in human and rat renal interstitium and can attenuated fibrosis by activation of hypoxia-inducible factor-α. Col2A1 and tropomyosin 1 could directly target the miR-29c. Liu and coworkers found that deletion of Smad7 promotes angiotensin II-mediated renal fibrosis and inflammation via Sp1-TGF-β1/Smad3-nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway that finally decrease the miR-29 expression.

Recently, Jiang and colleagues demonstrated that Sp1, which is a transcription factor and regulates the expression of several fibrosis-related genes, is a downstream target of miR-29c and regulating type I collagen production in tubular epithelial cells. Knockdown of miR-29c could sufficiently induce Sp1 and increase type I collagen expression. Transforming growth factor-β1 inhibits expression of the miR-29 family and promotes the expression of ECM components. Pharmacologic modulation of these microRNAs may have therapeutic potential in renal fibrosis.

**MiR-21.** MiR-21 is one of the most extensively investigated microRNAs and regulates the progression of renal fibrosis by targeting different genes including collagen I, fibronectin, and α-smooth muscle actin. Transforming growth factor-β1 positively regulates miR-21 expression by Smad3 and negatively by Smad2. Transforming growth factor-β1/Smad3 signaling is essential for miR-21 synthesis and enhancing posttranscriptional processing of pri-microRNA into pre-microRNA by Droscha. Conversely, miR-21 leads to amplification of TGF-β signaling by inhibition
Elevated levels of miR-21 leads to inhibition of lipid metabolism and increased oxygen radical production.\textsuperscript{87,88} Preserved expression of proximal tubule peroxisome proliferator-activated receptor \( \alpha \) (a regulator of lipid metabolism) attenuated interstitial inflammation and fibrosis in a mouse model.\textsuperscript{89} Wang and colleagues reported that miR-21 is involved in renal fibrosis in diabetic nephropathy mice by increasing tissue inhibitor of metalloproteinase-1 and decreasing of matrix metalloproteinase-9 proteins, which finally leads to increased deposition of ECM components.\textsuperscript{90} Deyand associates, indicated that miR-21 is involved in activation of mammalian target of rapamycin complex 1, which targets phosphatase and tensin homolog, in response to the TGF-\( \beta \) in murine mesangial cells. Therefore, TGF-\( \beta \)-stimulated miR-21 expression regulates hypertrophy of mesangial cells.\textsuperscript{91}

Knockdown of renal miR-21 restores Smad7 levels and suppresses activation of the TGF-\( \beta \) and nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathways, suggesting that miR-21 regulates renal injury by targeting Smad7.\textsuperscript{92} Suppression of miR-21 by a short hairpin RNA halted the progression of renal fibrosis in a mouse model of obstructive nephropathy.\textsuperscript{82} These findings clearly showed that miR-21 was a promoter of renal fibrosis and its inhibition might be an effective therapeutic option for suppression of fibrotic events in different renal diseases, including diabetic nephropathy, chronic glomerulonephritis, and chronic allograft dysfunction.\textsuperscript{82,87,92}

**Other MicroRNAs Involved in Renal Fibrosis**

High glucose media upregulate the miR-377 in cultured human and mouse mesangial cells and lead to reduced activity of P21-activated kinase 1 and superoxide dismutase 1/2, which finally enhance susceptibility to oxidant stress and accumulation of fibronectin.\textsuperscript{93} Macconia\textasciitilde and associates found an upregulation of miR-324-3p in rats models of progressive nephropathy, and it was associated with reduced expression of prolyl endopeptidase, a serine peptidase involved in the metabolism of angiotensin. Angiotensin-converting enzyme inhibitor downregulated miR-324-3p at glomerular and tubular levels. These data suggested that the protective effect of angiotensin-converting enzyme inhibitors may be due to the modulation of the miR-324-3p/prolyl endopeptidase pathway.\textsuperscript{94} MiR-382 can facilitate TGF-\( \beta \)-1-induced loss of renal epithelial characteristics.\textsuperscript{95} In vivo profibrotic effects of miR-382 have been shown to be through targeting and reduction of kallikrein 5, a proteolytic enzyme involved in degradation of extracellular matrix proteins. Knockdown of miR-382 demonstrated the important contribution of miR-382 to the inner medulla extracellular matrix abundance and interstitial fibrosis in mouse kidney.\textsuperscript{96} It is also reported that miR-150 promotes renal fibrosis by increasing profibrotic molecules through downregulation of antifibrotic protein suppressor of cytokine signaling 1 in lupus nephritis biopsy.\textsuperscript{97}

**MICRONRNA AS BIOMARKER AND THERAPEUTIC TARGET**

Recent progress in microRNA research has created a great promise to identify new diagnostic biomarkers and therapeutic targets in renal fibrosis. MicroRNAs are stable in tissues and biological fluids. Even after long-term room temperature storage and after multiple freeze-thaw cycles. This is because of their small size and packaging within exosomes. These properties make them a good candidate for detection and monitoring of renal fibrosis.

**Identified and Validated MicroRNAs in Chronic Allograft Dysfunction With Interstitial Fibrosis and Tubular Atrophy (IFTA)**

<table>
<thead>
<tr>
<th>MicroRNAs Method</th>
<th>Source Sample Size</th>
<th>Control Group</th>
<th>Type of Donor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>56 differentially expressed microRNAs</strong></td>
<td>Biopsy</td>
<td>27 cases (19 IFTAs, 8 controls) Urine 14 cases (7 IFTAs, 7 controls)</td>
<td>Stable normal allografts</td>
<td>Deceased 98</td>
</tr>
<tr>
<td>Up: miR-142-3p, miR-32, Down: miR-204, miR-107, miR-211</td>
<td>Microarray</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>33 differentially expressed microRNAs</strong></td>
<td>Biopsy</td>
<td>18 cases (10 IFTA, 8 controls)</td>
<td>Normal biopsies</td>
<td>Deceased and living 73</td>
</tr>
<tr>
<td>Up: miR-21, 142-3p, and 5p and the cluster comprising miR-506 Down: miR-30b and 30c</td>
<td>Deep sequencing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>50 differentially expressed microRNAs</strong></td>
<td>Serum 42 cases</td>
<td>Normal human kidneys</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Up: miR-21</td>
<td>Microarray</td>
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</tbody>
</table>

This table lists the identified and validated microRNAs in chronic allograft dysfunction with interstitial fibrosis and tubular atrophy (IFTA). The microRNAs include miR-142-3p, miR-32, miR-204, miR-107, and miR-211, which are upregulated in renal fibrosis. Additionally, miR-21, 142-3p, and 5p, as well as the cluster comprising miR-506, are downregulated in chronic allograft dysfunction. The microRNAs are detected in biopsy samples of kidney tissue, and the results indicate potential biomarkers for diagnostic and therapeutic targets.
the diseases.\textsuperscript{10,47} In the course of kidney allograft fibrosis, miR-142-3p/5p, miR-204, miR-107, miR-211, miR-21, miR-32, and miR-30, all are upregulated at the tissue level and could be considered as biomarkers. In the course of IFTA, urinary miR-142-5p increases and miR-204 decreases more than 300-fold (Table).

There is also considerable interest in studying exosomal microRNA in biological fluids.\textsuperscript{10,63,99} Blockade of TGF-\(\beta\) signaling by Smad7 prevents experimental renal fibrosis through regulating TGF-\(\beta\)/Smad3-mediated renal expression of miR-21, miR-192, and miR-29b.\textsuperscript{86} Taken together, overexpression of renal Smad7, which restores the balance of TGF-\(\beta\)/Smad signaling, and restoring miR-29, miR-200, and miR-30 and suppressing miR-21, miR-192, and miR-324-3p could be a novel translational approach for treatment of renal fibrosis.

CONCLUSIONS
Both diagnosis and intervention of chronic allograft nephropathy are a great challenge for transplant scientists, and finding a noninvasive biomarker of allograft monitoring before the development of a clinical phenotype is the paramount goal in kidney transplantation. The increasing information about microRNAs (78 hits for “microRNA” and “kidney fibrosis” keyword in PubMed up to August 2013) suggests their crucial roles in progression of graft fibrosis (Figure 2). MicroRNAs regulate the biological pathways of IFTA by targeting varieties of mRNAs. Using microRNAs as a promising novel diagnostic markers and also therapeutic targets offers new avenues in allograft research.

CONFLICT OF INTEREST
None declared.

REFERENCES
3. Nankivell BJ, Chapman JR. Chronic allograft nephropathy:

![Figure 2. MicroRNAs in the pathophysiology of renal fibrosis.](image-url)
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