

# Downregulation of Profibrotic Gene Expression by Angiotensin Receptor Blockers

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**Introduction.** Chronic allograft nephropathy is characterized by interstitial fibrosis and tubular atrophy. The main players in the process of fibrosis are transforming growth factor- $\beta$  (TGF- $\beta$ ) and miR-21 expression with a bidirectional interplay. This study aimed to evaluate the effects of angiotensin receptor type 1 antagonist, losartan, on peripheral blood and tissue expression of TGF- $\beta$  and miR-21 and histologic findings in allograft biopsy in kidney transplant recipients.

**Materials and Methods.** In a randomized controlled trial, 54 patients were enrolled and divided randomly into 2 groups. Group 1 was treated with a daily dose of 25 mg of losartan and group 2 was considered as control. Blood sampling was done at 48 hours posttransplantation and the 3rd and 6th months after transplantation for measurement of TGF- $\beta$  RNA and miR-21. Protocol biopsy was performed at the 6th month posttransplantation for RNA extraction and histologic evaluation of interstitial fibrosis and tubular atrophy.

**Results.** Although patients were not different initially, those who underwent treatment with losartan had lower miR-21 and TGF- $\beta$  levels in circulating PBMCs, and there was a decreasing trend in peripheral blood TGF- $\beta$  levels during the 6-month follow-up period. Tissue expression of miR-21 and TGF- $\beta$  was also considerably lower among the losartan-treated patients at the time of tissue biopsy.

**Conclusions.** Losartan treatment decreased the tissue expression of miR-21 and TGF- $\beta$  and tissue fibrosis in kidney transplant patient, and it had a protective effect on allograft function and may delay chronic allograft dysfunction by reducing mediators of fibrosis.

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## INTRODUCTION

Despite improvements in immunosuppressive therapy and better short-term survival of kidney allografts, long-term survival does not improve. Chronic allograft nephropathy is the major pathologic finding in late-onset allograft dysfunction.<sup>1</sup> A wide range of factors have been proposed in the pathogenesis of chronic allograft nephropathy, including chronic immunologic insults, calcineurin inhibitor toxicity, and epithelial to mesenchymal transition. Independent of the etiology, chronic allograft nephropathy is

characterized by interstitial fibrosis and tubular atrophy (IF/TA), the term which substituted chronic allograft nephropathy.<sup>2,3</sup> The major player in the process of fibrosis is transforming growth factor- $\beta$  (TGF- $\beta$ ), the signaling of which results in fibroblast proliferation, epithelial to mesenchymal transition, and extracellular matrix expansion. Recent data in evaluation of TGF- $\beta$  pathways demonstrated the role of micro-RNAs (miRNA) in its control.<sup>4,5</sup>

Micro-RNAs are 18 to 24 nucleotides noncoding RNA that control gene transcription by degradation or inhibition of RNA transcription.<sup>6</sup> Numerous

miRNAs have been implicated in the pathogenesis of TGF- $\beta$ -induced fibrosis, such as miR-21, members of both the miR-200 and miR-29 families, miR-192, miR-216, miR-217, miR-377, miR-93 and miR-382.<sup>6</sup> Micro-RNA-21 has been demonstrated to be upregulated after acute kidney injury, and its prolonged overexpression causes tissue fibrosis. Transforming growth factor- $\beta$  signaling results in miR-21 expression, which in turn, by inhibiting Smad-7 (endogenous negative regulator of TGF- $\beta$  pathway), enhances the TGF- $\beta$  signaling.<sup>4,7</sup> Micro-RNA-21 is upregulated in fibroblasts that activated in renal fibrosis.<sup>8</sup> Micro-RNA-21 also enhances the oxidative injury in the kidneys and contributes to tissue injury and fibrosis via downregulation of peroxisome proliferator-activated receptor- $\alpha$ .<sup>9</sup>

Transforming growth factor- $\beta$  acts via its receptors; TGF- $\beta$  receptor type I and type II and angiotensin II have a dual effect on TGF- $\beta$ . On the one hand, angiotensin II induces TGF- $\beta$  and on the other hand, via its type 1 receptor, enhances the expression and activity of receptor type I. On the contrary, angiotensin II reduces the expression of receptor type II by acting on its angiotensin type 2 receptor.<sup>10</sup> There are reports on attenuation of TGF- $\beta$  production in the allograft with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. The latter have been demonstrated to normalize the plasma level of TGF- $\beta$  in kidney transplant patients and regenerate kidney vasculature.<sup>11,12</sup>

Considering the protective effects of angiotensin type 2 receptor, in this study, we evaluated the effects of angiotensin type 1 receptor antagonist (losartan) on peripheral blood and tissue expression of TGF- $\beta$  and miR-21 and histologic findings in allograft biopsy in kidney transplant recipients.

## MATERIAL AND METHODS

### Patients and Data Collection

In a randomized clinical trial, 78 kidney transplant recipients were evaluated for enrolment in the study, from November 2013 to December 2015, at Labbafinejad Hospital. The ethic committee of Chronic Kidney Disease Research Center, Shahid Beheshti University of Medical Sciences, approved the study protocol. This clinical trial was registered with the ISRCTN registry (ISRCTN15884223). Informed consent was obtained from all participants.

Patients older than 18 years who received their

first kidney transplantation from a living donor and their underlying kidney disease were diabetes mellitus, hypertension, and glomerulonephritis were evaluated. Those with normal graft function at the end of first week of transplantation and without a known history of allergy or intolerance to losartan were enrolled.

Patients receiving a second transplant or cadaveric transplantation, those with delayed graft function or acute rejection during the first week after transplantation, those with BK virus-associated nephropathy and urinary tract obstruction, and those who were unwilling to participate were excluded.

With a concealed allocation method, we randomized kidney transplant candidates into 2 groups and they were matched for the underlying illness. Group 1 was treated with a daily dose of 25 mg of losartan and group 2 did not receive medication other than those for the standard care (controls). In case of necessity for antihypertensive drugs in the control group, drugs other than angiotensin-converting enzyme inhibitors and angiotensin receptor blockers were used. Both groups were treated for hypertension with calcium channel blockers, if necessary. All of the patients were treated with the same immunosuppressive protocol of cyclosporine, mycophenolate mofetil, and prednisolone.

Blood sampling was done at 48 hours posttransplantation, on the 3rd month and 6th months after transplantation, for measurement of TGF- $\beta$  RNA and miR-21. Protocol biopsy was performed at the 6th month posttransplantation for RNA extraction and histologic evaluation of IF/TA. Allograft biopsies were reported by one pathologist who was unaware of study groups and based on the Banff 2009 classification.

Demographic data, blood pressure measurements, and laboratory testing including serum creatinine, potassium, fasting blood glucose, and cytomegalovirus and BK virus viremia, were recorded weekly for the 1st month and biweekly for the 2nd month after transplantation, and then monthly for the rest of the study. Estimated glomerular filtration rate (GFR) was calculated using the 2009 CKD-EPI creatinine equation.

### Tissues and Cells RNA Extraction

Whole blood samples (10 mL) were collected in ethylenediaminetetraacetic acid. Peripheral

blood monocyte cells (PBMCs) were isolated by a standard Ficoll density gradient centrifugation. The isolated PBMCs were stored in RNAlater solution at 4°C for 1 night and then stored at -80°C till RNA extraction. Two cores of biopsy samples were taken from each patients. One was formalin-fixed and paraffin-embedded and used for histologic evaluation and the other was stored in RNAlater solution at 4°C for 1 night and then stored at -80°C till RNA extraction. The mirVana miRNA Isolation Kit was used to extract RNAs (TGF- $\beta$  and miR-21) from both PBMCs and tissue samples. Gene expression in tissue and blood samples were evaluated by quantitative real-time polymerase chain reaction and TaqMan Micro-RNA Assays (Applied Biosystems, Foster city, USA).

### Statistical Analysis

Expression levels of miRNA was calculated based on relative threshold cycle number:

Relative expression =  $2 - [(cycle\ threshold\ difference\ of\ case) - (cycle\ threshold\ difference\ of\ control)]$

Nonparametric analysis was done using the Spearman correlation test and the Mann-Whitney test. Data were analyzed using the SPSS software (Statistical Package for the Social Sciences, version 21.0, IBM Corp, New York, NY, USA).

### RESULTS

From November 2013 to December 2015, 78 candidates of first living kidney transplantation were evaluated for enrollment. Twenty-four patients were excluded based on previously described criteria: 5 due to delayed graft dysfunction, 4 due to change in immunosuppressive regimen, 10 due to unwillingness to participate, 2 due to patients' death, and 3 due to nephrectomy. Fifty-four patients were enrolled and divided randomly into 2 groups. During the follow-up period, 8 patients dropped out of study due to recurrent focal-segmental glomerulosclerosis (n = 1), BK virus nephropathy (n = 1), acute rejection (n = 2), and noncompliance (n = 4). Finally, 19 patients in the losartan group and 27 in the control group completed the study period (Figure 1). Only 26 patients underwent

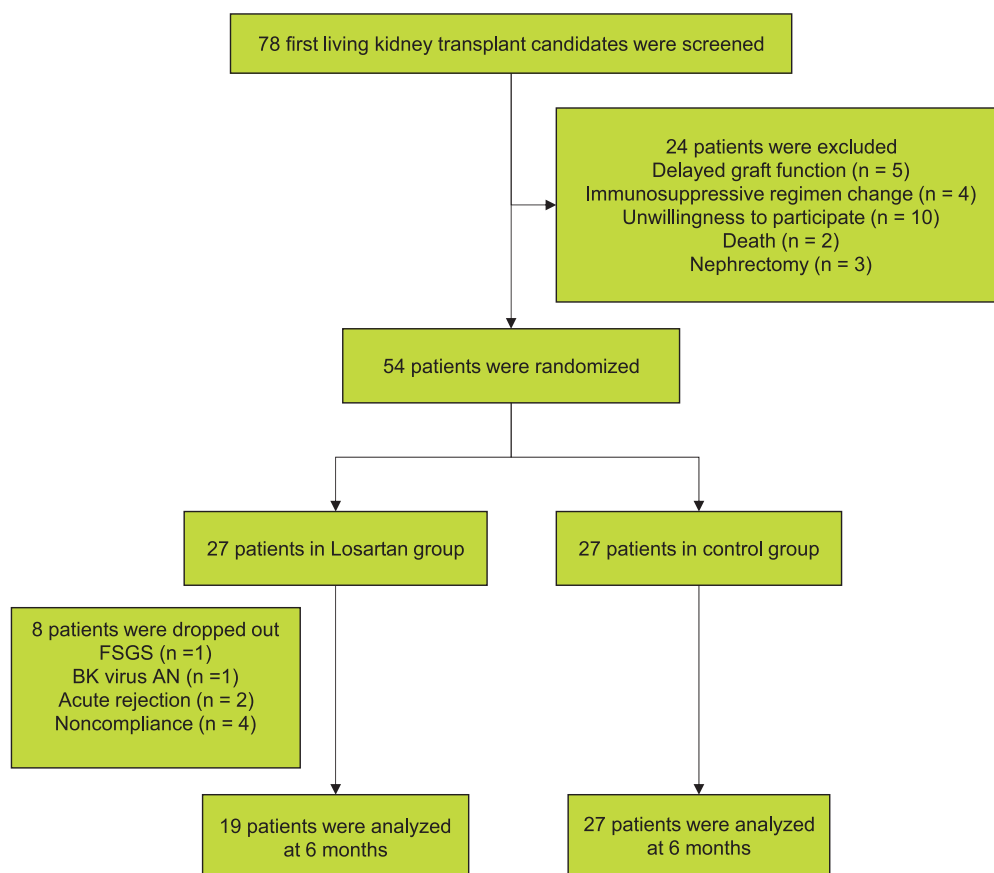


Figure 1. Study design.

protocol biopsy at 6 months after transplantation.

The mean age of the patients in losartan group was  $42.84 \pm 9.2$  years and in the control group was  $35.22 \pm 10.4$  years ( $P > .05$ ). The mean donor age was not significantly different between the groups either (31.47 years versus 26.66 years;  $P > .05$ ).

Considering the antihypertensive effect of losartan and in order to exclude the consequences of blood pressure control on allograft survival and fibrosis, systolic and diastolic blood pressures were compared between the study groups. There was no significant difference in baseline and the follow-up measurements among the patients. ( $P > .05$ ; Table 1).

There was no significant differences in blood pressure, estimated GFR, serum potassium level, hemoglobin level, and cyclosporine level between the two groups (Table 2). The difference in estimated GFR was not significant even at 12 months after transplantation. (72.7 mL/min versus 70.9 mL/min in the case and control groups, respectively;  $P > .05$ ; Figure 2).

The miR-21 levels in PBMCs were lower among the patients treated losartan, although the difference was only evident after 6 months of treatment (0.855- versus 1.560-fold change in the case and control

groups, respectively;  $P < .05$ ; Table 3).

Of 26 patients who participated in protocol biopsy at month 6 posttransplantation, 11 were from losartan group and 15 were in the control group. Assessing tissue expression of miR-21 demonstrated a significantly less amount of miR-21 in biopsy samples of patients treated with losartan (0.861- versus 2.376-fold change in the case and control groups, respectively;  $P < .05$ ; Table 4).

Although the two groups were not different at baseline (6.725- versus 6.938-fold change in the case and control groups, respectively;  $P > .05$ ), TGF- $\beta$  level in PBMCs was significantly lower 3 months and 6 months after initiation of losartan (Table 3). Tissue expression of TGF- $\beta$  was significantly less in kidney biopsy specimens of the patients who were treated with losartan (4.317- versus 10.782-fold change in the case and control groups, respectively;  $P < .05$ ; Table 4).

In order to evaluate the effect of losartan of IF/TA, the marker of chronic graft dysfunction, biopsy samples were evaluated and scored by a single and blinded pathologist. Despite of the fact that IF/TA was lower in the losartan-treated group, the difference was not significant. ( $8.57 \pm 2.3\%$  versus  $10.88 \pm 1.2\%$ ;  $P > .05$ ; Table 4).

**Table 1.** Demographic Data and Baseline Characteristics of Patients\*

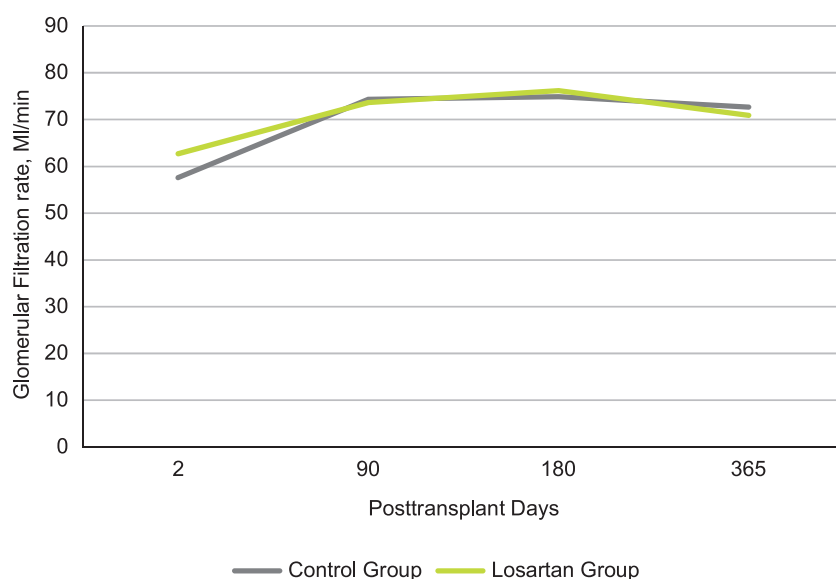
Variable	Case Group (n = 19)	Control Group (n = 26)	P
Male sex	14 (73.0)	12 (46.0)	< .05
Recipient age, y	$42.84 \pm 10.10$	$35.22 \pm 12.30$	> .05
Donor age, y	$31.47 \pm 9.20$	$26.66 \pm 10.60$	> .05
Systolic blood pressure, mm Hg	$97 \pm 22$	$89 \pm 25$	> .05
Diastolic blood pressure, mm Hg	$78 \pm 13$	$73 \pm 15$	> .05
Glomerular filtration rate, mL/min	$62.7 \pm 10.1$	$57.6 \pm 12.4$	> .05
Serum potassium, mEq/L	$4.5 \pm 1.1$	$6.4 \pm 0.9$	> .05
Fasting blood glucose, mg/dL	$115 \pm 12$	$104 \pm 15$	> .05
Hemoglobin, g/dL	$10.6 \pm 1.1$	$9.0 \pm 2.4$	< .05

\*Values are mean  $\pm$  standard deviation except for male sex, which is frequency (percentage).

**Table 2.** Changes in Studied Parameters in the Case and Control Groups\*

Parameters	Case Group		Control Group		P
	Month 3	Month 6	Month 3	Month 6	
Systolic blood pressure, mm Hg	$123 \pm 12$	$125 \pm 10$	$120 \pm 13$	$124 \pm 15$	> .05
Diastolic blood pressure, mm Hg	$76 \pm 7$	$75 \pm 7$	$75 \pm 8$	$75 \pm 5$	> .05
Glomerular filtration rate, mL/min	$73.6 \pm 5$	$76.2 \pm 4.6$	$74.3 \pm 5.3$	$74.9 \pm 4.7$	> .05
Serum potassium, mEq/L	$4.7 \pm 0.8$	$4.4 \pm 1$	$4.4 \pm 0.9$	$4.3 \pm 0.5$	> .05
Hemoglobin, g/dL	$11.8 \pm 1.1$	$12.7 \pm 0.9$	$11.3 \pm 1.2$	$13.2 \pm 0.7$	> .05
Cyclosporine level C0	234	204	254	210	> .05
Cyclosporine level C2	823	400	835	370	> .05

\*Values are mean  $\pm$  standard deviation or median.



**Figure 2.** Estimated glomerular filtration rate trend during 1-year follow-up.

**Table 3.** Micro-RNA-21 and Transforming Growth Factor- $\beta$  Levels in Peripheral Blood Monocyte Cells

Parameter	Case Group			Control Group		
	48 Hours	3 Months	6 Months	48 Hours	3 Months	6 Months
Micro-RNA-21	0.645	0.736	0.855*	0.662	1.029	1.560*
Transforming growth factor- $\beta$	6.725	5.379*	4.546*	6.938	6.727*	7.752*

\* $P < .05$

**Table 4.** Tissue Expression of Micro-RNA-21 and Transforming Growth Factor- $\beta$  and Histologic Findings in 6-months' Protocol Biopsy

Parameter	Case Group (n = 11)	Control Group (n = 15)	P
Micro-RNA-21	0.861	2.376	< .05
Transforming growth factor- $\beta$	4.317	10.782	< .05
Interstitial fibrosis/ tubular atrophy	8.57	10.88	> .05

The two groups were not significantly different with respect to histologic findings. There was no significant correlation between blood level and tissue expression of TGF- $\beta$  and miR-21 with pathologic findings including IF/TA.

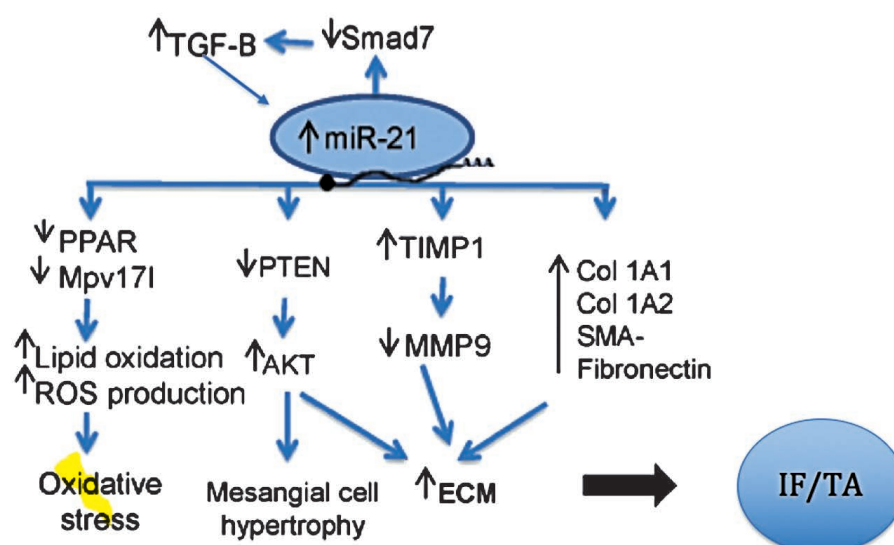
### Safety of Losartan

During the first 6 months of losartan use, there was no significant increase in the incident of hyperkalemia or worsening of anemia in the intervention group (Table 2).

### DISCUSSION

The interaction between miR-21 and TGF- $\beta$  and the consequent activation of downstream pathways of oxidative stress, mesangial hypertrophy and increased extracellular matrix is a part of the

network that results in tissue fibrosis (Figure 3). Numerous studies demonstrated the diminishing effect of losartan on plasma levels of TGF- $\beta$  and tissue expression of its receptor.<sup>9,13-14</sup> In this study, we evaluated the role of losartan in breaking the amplifying cycle between miR-21 and TGF- $\beta$ , and its effect on allograft fibrosis. Though patients were not different initially, those underwent treatment with losartan had lower miR-21 and TGF- $\beta$  levels in circulating PBMCs, and there was a decreasing trend in peripheral blood TGF- $\beta$  levels during the 6-month follow-up. Tissue expression of miR-21 and TGF- $\beta$  was also considerably lower among losartan treated patients at the time of tissue biopsy. As miR-21 and TGF- $\beta$  have been extensively studied as mediators of kidney fibrosis,<sup>4,8,15,16</sup> we expected that patients who were treated with losartan had



**Figure 3.** The interaction of micro-RNA-21 and transforming growth factor- $\beta$  in inducing interstitial fibrosis/ tubular atrophy (adapted with permission from Zununi Vahed, et al. Iran J Kidney Dis. 2014;8:4-12).<sup>1</sup>

less amount of IF/TA in their biopsy samples. However, there was no significant differences in IF/TA scoring. This finding might be due to the fact that biomarker changes are occurring in advance of histologic changes. A drop in the losartan group is further due to concern from colleagues about the side effects of the drug and the patient's lack of follow-up.

As the incidence of IF/TA increased by the time elapsed after transplantation,<sup>1</sup> and there is no solid correlation between the pathologic findings and estimated GFR, protocol biopsy after 1 year posttransplantation might make effect of losartan on tissue fibrosis evident. The decreasing trend of TGF- $\beta$  may support this fact.

### CONCLUSIONS

Apart from its role in hemodynamics of glomerular capillary, losartan has a protective effect on allograft function and may delay CAD by reducing mediators of fibrosis. A larger clinical trial with higher doses of losartan and longer follow-up and kidney biopsy of all patients might help to validate these findings.

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### CONFLCIT OF INTEREST

None declared.

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