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Association of MicroRNA146a G>C and MicroRNA196a-2 C>T Gene Polymorphisms with Outcome of Kidney Transplantation in Iranian Patients

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ABSTRACT

Acute organ rejection remains a serious clinical challenge. Novel accessible biomarkers of acute rejection could easily enable us to detect the rejection earlier and make more fine-tuned calibration of immunosuppressive or new target treatment possible. Control of gene expression by microRNAs influences many cellular functions, including cellular differentiation, cell proliferation, cell development, and functional regulation of the immune system. Therefore, this study was aimed to investigate if miRNA146a G>C and miRNA196a-2 C>T gene polymorphisms are associated with kidney transplant rejection in Iranian patients.

Tissue samples were collected from 100 renal transplant patients between the years 2009 and 2013. The miRNA146a G>C (rs2910164) and miRNA196a-2 C>T (rs11614913) gene polymorphisms were evaluated in kidney transplant patients; using the in-house-polymerase chain Reaction-restriction fragment length polymorphism (PCR-RFLP) method.

In this study, we found that the CC genotype, C and G alleles of the miRNA146a G>C polymorphism was associated with increased risk of transplant rejection in kidney transplant patients (p=0.003, p=0.01 and p=0.01), respectively. The CC genotype, T, and C alleles of the miRNA196a-2 C>T were also significantly more frequent in transplanted patients compared to healthy controls (p=0.02, p=0.05, and p=0.05), respectively. However, significant associations were not found between miRNA196a-2 C>T polymorphisms and kidney transplant rejection.

The CC genotype, G, and C allele of the miRNA146a G>C and also, the CC genotype, T and C alleles of the miRNA196a-2 C>T may be genetically susceptible factors for transplant rejection and development of kidney disorders, especially in Iranian patients. Further studies are required to validate these findings in a larger population, as well as in patients with different ethnic origins.

Keywords: Kidney transplantation; MicroRNAs; Polymorphism

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INTRODUCTION

Kidney transplantation is the best choice for the treatment of patients suffering from end-stage renal disease (ESRD).[1] Despite progress in the use of immunomodulatory drugs, acute rejection and mortality remain as serious complications after transplantation.[2] The patients receiving transplantation require long-term therapy with non-specific, toxic, and immunosuppressive drugs, which may result in the loss of allografts.[3] Therefore, there is an immediate need to introduce a safe and noninvasive protocol for the early detection of patients susceptible to acute rejection.

MicroRNAs (miRNAs) are small (~19–24 nucleotides) noncoding RNA molecules that have an essential role in the regulation of gene expression.[4-7] Recently, it has been shown that miRNAs regulate some features of cellular and molecular processes including inflammation, immune system, signaling pathways, and physiological and pathophysiological processes involved in allograft rejection and acute kidney injury.[8-9] Expression of different miRNAs in the body fluids and tissues probably reflects allograft state after transplantation.[10] Some studies indicated that following kidney transplantation the amount of miRNAs changes, and may contribute to graft loss.[11-12]

Several studies were performed to clarify the molecular mechanisms of acute rejection for the early detection of patients susceptible to acute rejection.[13-14] Recently, current studies focus on the correlation between kidney transplantation and miRNAs polymorphisms.[15-16]

Single nucleotide polymorphisms (SNPs), is a type of genetic mutation which leads to a difference in genetic context in various populations and may have a role in initiating and complicating clinical outcomes especially in transplant patients.[17-19]

SNPs also could regulate miRNAs expression and thereby probably contribute to the induction of hundreds of disorders.[20] Some studies indicate that SNPs in miRNA146a G>C and miRNA196a-2 C>T are associated with kidney disorders.[21-22] A previous study demonstrated that Asian populations are more susceptible to ESRD compared to European ones. Meta-analysis studies also showed that the expression pattern of miRNA196a-2 C>T and miRNA146a G>C are different among Asian and European populations. CC genotype and T allele of miRNA196a-2 C>T are variant forms in Eastern Asia. CC genotype and C allele of miRNA146a G>C also is more frequent in Asian populations.[23-24] Therefore, in this study, the correlation between kidney transplant rejection and miRNA196a-2 C>T and miRNA146a G>C polymorphisms was investigated in the Iranian population.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Grant number: 92-196). Written informed consent was obtained from all patients and healthy controls. All participants were Iranian.

Study Population

In this study, a total of 100 patients, who had undergone kidney transplantation at the transplant ward of Namazi hospital, affiliated to Shiraz University of Medical Sciences, Shiraz, Iran, were consecutively enrolled between 2009 and 2013. Patients were followed up for transplantation outcomes and acute rejection events for at least six months after transplantation. These patients were divided into two groups according to the presence or absence of acute rejection (AR and non-AR group/control). Thirty-four out of 100 (34%) patients experienced acute rejection. The control group consisted of 220 individuals randomly selected from the Blood Transfusion Organization Center, Shiraz, Iran. Demographic information is shown in Table 1.

Table 1. Demographic characteristics of kidney transplant patients

Underlying disease	Patients N (%)	Rejected patients N (%)	Non-Rejected patients N (%)
Diabetes	26(26)	4(11.8)	22(33.3%)
Polycystic	7(7)	-	7(10.6%)
Other (kidney failure)	67(67)	30(88.2)	37(56.1%)
Total	100	34	66

N: Number

DNA Extraction

Tissue samples from patients were obtained from the sampling center of Shiraz Transplantation Research Center and blood samples were secured from Shiraz Blood Transfusion Center to be used as a control group. DNA was isolated from tissue samples; using the DNPTM DNA purification kit (Cinna Gene, Iran) according to the manufacturer's instruction. Isolation of DNA from whole blood of healthy control was done using Phenol-chloroform (Cinna Gene, Iran) extraction method as indicated by the manufacturer.

Polymerase Chain Reaction-restriction Fragment Length Polymorphism (PCR-RFLP) Method

The SNPs of miRNA146a G>C (rs2910164) and miRNA196a-2 C>T (rs11614913) genes were analyzed by RFLP method using a thermal cycler (Eppendorf, Germany). Items are summarized in Table 2, included: fragment sizes, product lengths, primers, and restriction enzymes. For confirmation of successful PCR amplification, the PCR product was run on a 1.5% agarose gel. Twelve µL of each PCR product was digested with 5 units of restriction enzymes (indicated under results) and the products were run on a 3% agarose gel and directly visualized under ultraviolet illumination.

Statistical Analysis

Direct gene counting was used for the calculation of the allele and genotype frequencies in patient and control groups. Statistical evaluation was carried out using SPSS for windows ver.16 (SPSS Inc, Chicago, IL, USA), and Epi Info (CDC, Atlanta, USA) software was used for statistical evaluation. The frequencies of the alleles/genotypes were compared in cases and controls by $\chi 2$ test or Fisher's exact test when appropriate. Relative risks, odds ratios, and 95% confidence intervals (CIs) were estimated. The statistically significant *p*-value was considered less than 0.05 and calculated by a two-tailed method.

RESULTS

Demographic Profiles and Clinical Features

Among 100 consecutive recipients, 78.3% were males and 21.7% were females. The male to female ratio was 19.3 in the rejected group and 57.21 in others. The age range in all patients was 11-68 years with a mean of 43.2±12.3 years and 45.2±10.3 and 45.2±12.3 years in rejected and Non-rejected groups, respectively. The most frequent age range in patients was between 45 to 63 years (Table 3). In the present study, 6% of the recipients received the graft from living

Table 2. Conditions of the genes: The primers, fragment size, restriction enzymes, amplification, and polymerase chain reaction (PCR) mixture

Genes (variants)	Primer sequence (5′–3′)	Fragment sizes (bp)	Restriction enzymes	Thermocycler conditions	PCR mixture conditions
miRNA146a G>C	F: 5′-CATGGGTTGTGTCAGTGTCAGAGCT-3′ R:5′-TGCCTTCTGTCTCCAGTCTTCCAA-3′	GG=147C G=147, 122, 25 CC=122, 25	SacI (5′-G A G C T↓C-3′)	95°C/5 min, 40 Cycle (95°C/1 min, 59 and 61°C/1 min,72°C /1 min), 72°C/5 min	D.W=10 PCR buffer=2.5 (10 X) MgCl2=0.75 (50 mM) dNTP=0.5 (10 p moL/L) Forward primers=0.5 (10 p moL/L) Reverse primers=0.5 (10 p moL/L) Tag DNA polymerase=0.25 (5 unit/µl) DNA=10
miRNA196a-2 C>T	F:5′-CCCCTTCCCTTCTCCTCCAGATA-3 R:5′-CGAAAACCGACTGATGTAACTCCG-3′	TT=149 CT=149, 125, 24 CC=125, 24	MspI (5′-C↓C G G-3′)		D.W=15 PCR buffer=2.5 (10X) MgCl2=0.75 (50 mM) dNTP=0.5 (10 p moL/L) Forward primers=0.5 (10 p moL/L) Reverse primers=0.5 (10 p moL/L) Tag DNA polymerase=0.25 (5 unit/µL) DNA=5

donors while 94% took their grafts from cadavers. The control group consisted of 220 individuals, with 68.9% males and 31.1% females. The age range was between 30 to 60 years with a mean of 44.1±8.2 years.

Alleles and Genotypes Frequencies

Alleles and genotypes frequencies for miRNA146a G>C, and miRNA196a-2 C>T were determined in the rejected and non-rejected groups of kidney transplant recipients. The results of agarose gel electrophoresis of studied microRNA gene polymorphisms are presented in Figure 1.

Study of miRNA146a G>C and miRNA196a-2 C>T Polymorphisms in Patients and Healthy Controls

The CC genotype, T and C alleles of the miRNA196a-2 C>T (rs11614913) polymorphism is associated with higher increasing risk in kidney transplant patients (OR=2, 95%CI:1.05-3.79, *p*=0.02; OR=0.72, 95%CI:0.51-1.02, *p*=0.05; OR=1.39, 95%CI:0.98-1.97, *p*=0.02, respectively).

However, genotypes and alleles of the other studied miRNA polymorphisms had no significant effect on the outcomes of kidney transplant recipients (Table 4).

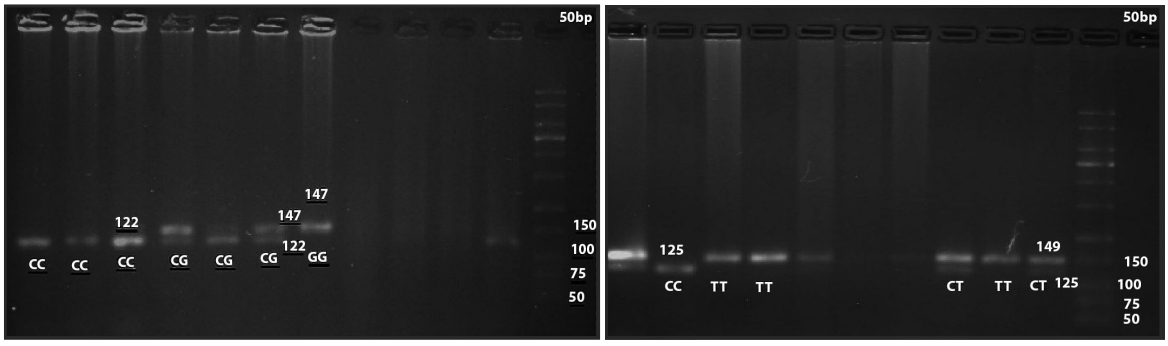
Inheritance of miRNA146a G>C and miRNA196a-2 C>T genes in Transplant Recipients

The CC genotype, G and C alleles of the miRNA146a G>C (rs2910164) polymorphism is associated with increased risk of transplant rejection in kidney transplant patients (OR=3.92, 95%CI:1.36-11.45, *p*=0.003; OR=0.48, 95%CI:0.26-0.92, *p*=0.01; OR=2.06, 95%CI:1.09-3.91, *p*=0.01, respectively). However, genotypes and alleles of the other studied miRNA polymorphisms had no significant effect on the outcomes of kidney transplant recipients (Table 5).

Table 3. Demographic characteristics of kidney transplant patient

Characteristic	Rejected group N (%)	Non-rejected group N (%)
Gender		
Male	30(88)	51(77)
Female	4(12)	15(23)
Type of transplant		
Cadaver	32(94)	60(91)
Living	2(6)	6(9)

N: Number



A **B**

Figure 1. The miRNA146a G>C genotype created by the SacI restriction enzyme (A) The miRNA196a-2 C>T genotype created by the MspI restriction enzyme (B)

Table 4. The allelic and genotypic abundance of miRNA146a G>C and miRNA196a-2 C>T polymorphisms, based on case and control

Locus	Genotype and Allele	Case N (%)	Control N (%)	*p*	OR	95% CI
miRNA146a G>C	GG	50(50)	100(45.4)	0.57	1.20	(0.7-1.98)
	GC	36(36)	80(36.4)	0.90	0.98	(0.58-1.66)
	CC	14(14)	40(18.2)	0.80	0.73	(0.36-1.48)
	G	136(68)	280(63.6)	1.15	1.21	(0.84-1.76)
	C	64(32)	160(36.4)	1.15	0.82	(3.57-1.19)
miRNA196a-2 C>T	TT	26(26)	70(31.8)	0.20	0.75	(0.43-1.32)
	CT	50(50)	120(54.6)	0.40	0.83	(0.50-1.38)
	CC	24(24)	30(13.6)	**0.02**	2	(1.50-3.79)
	T	102(51)	260(59.1)	**0.05**	0.72	(0.51-1.02)
	C	98(49)	180(40.9)	**0.02**	1.39	(0.98-1.97)

N: Number

Table 5. The allelic and genotypic abundance of miRNA146a G>C and miRNA196a-2 C>T polymorphisms in kidney transplants, based on rejected and non-rejected groups

Locus	Genotype and Allele	Reject N (%)	Non-Reject N (%)	*p*	OR	95% CI
miRNA146a G>C	GG	8(23.5)	22(33.3)	0.300	0.62	(0.21-1.73)
	CG	12(35.3)	34(51.5)	0.120	0.51	(0.20-1.31)
	CC	14(41.2)	10(15.2)	**0.003**	3.92	(1.36-11.45)
	G	28(41.2)	78(59.1)	**0.010**	0.48	(0.26-0.92)
	C	40(58.8)	54(40.9)	**0.010**	2.06	(1.09-3.91)
miRNA196a-2 C>T	TT	7(20.6)	20(30.3)	0.290	0.60	(0.20-1.75)
	CT	13(38.2)	30(45.5)	0.480	0.74	(0.29-1.87)
	CC	14(41.2)	16(24.2)	0.080	2.19	(0.83-5.83)
	T	27(32.1)	70(53)	0.070	0.58	(0.31-1.10)
	C	41(67.9)	62(47)	0.070	1.71	(0.91-3.25)

N: Number

DISCUSSION

Several studies demonstrated that miRNA's have a substantial role in the regulation of transplant rejection.[10-12] Therefore, since the expression of miRNA molecules may be affected by gene polymorphisms, in this study, we investigated the effects of two representative SNPs, the miRNA146a G>C rs2910164 and miRNA196a-2 C>T rs11614913 polymorphisms on the outcome of kidney transplantation in Iranian recipients.

In this study we did not observe any association of rs11614913 with kidney transplant rejection, However, compared to healthy controls, transplant recipients showed a higher frequency of rs11614913, suggesting that rs11614913 may have a substantial role in the progression of kidney disorders. rs11614913 is located on the 3′ mature sequence of SNP conversion or mutations may alter the expression pattern and function of miRNA196a-2 C>T which will affect several target genes.[25] Showed that polymorphism in miRNA196a-2 C>T is associated with tumor recurrence after liver transplantation.[26] A report by XD Li et al also indicated that miRNA196a-2 C>T polymorphism is associated with cirrhosis which leads to hepatocellular carcinoma.[27] C Zhang et al showed that miRNA196a-2 C>T could be used as a predictive biomarker for chronic kidney disorders.[28] Other studies also demonstrated a correlation between miRNA196a-2 C>T polymorphism and the risk of development of

cancers such as lung, breast, stomach, esophageal and colorectal cancers.[24,29-33] Such findings are in accordance with our results. We have demonstrated that the rs11614913 T allele variant and CC genotype present in kidney transplant recipients may provide a supportive microenvironment for kidney disorders compared to the healthy controls.

To date, the role of the miRNA146a G>C (rs2910164) polymorphism in kidney transplant rejection in the Iranian population has not been reported. Therefore, in this study, we investigated the correlation between the rs2910164 polymorphism with the risk of acute kidney rejection. In this study, we observed an association between rs2910164 and susceptibility to kidney transplant rejection in an Iranian population. Several studies indicated that there are correlations among miRNA146a G>C polymorphism and the progression of many disorders such as severe sepsis,[34] systemic lupus erythematosus,[35] chronic periodontitis,[36] and cancers.[37-39] Deng et al have shown that miRNA146a G>C polymorphism could increase the risk of metastasis.[40] Furthermore, H. Yang et al showed that miRNA146a G>C polymorphism is associated with the risk of classic Kaposi sarcoma.[41] Moreover, J Lin et al demonstrated that miRNA146a G>C polymorphism was associated with a higher rate of morbidity in biopsy-proven IgA nephropathy.[21] A study by N Stickel et al also indicated that G/C polymorphism of miRNA146a G>C is associated with the risk of improving severe acute GVHD after allogeneic hematopoietic cells transplantation.[42] Moreover, miRNA146a G>C polymorphism is associated with coronary artery disease and renal cancer.[43-44] The underlying mechanisms of miRNA146a G>C (rs2910164) polymorphism in the induction of various disorders are still unclear. However, genetic variation of miRNA146a G>C is associated with altered expression patterns which could alter the binding activity to target genes.[34,45-46] In this study, we observed a higher frequency of rs2910164 GC genotype in patients experiencing kidney transplant rejection. Therefore, we could conclude that the altered expression pattern of miRNA146a G>C probably affects several target genes and thereby interfere with their function and contributes to the development of kidney rejection in the Iranian population.

This study has demonstrated that patients with the miRNA CC genotype, T and C alleles showed an increased risk for kidney disorders compared with healthy controls. Our finding also demonstrated that the miRNA146a G>C polymorphism rs2910164 may serve as a new risk factor for the development of kidney transplant rejection in the Iranian population. Moreover, this study indicated that miRNA196a-2 C>T may have prediction potential for kidney disorders which leads to kidney transplantation. Further investigations are required to clarify underlying mechanisms to open new avenues for prediction, diagnosis, and treatment of several disorders.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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