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Research Article

Genetic polymorphisms of Interleukin-18 are not associated with allograft function in kidney transplant recipients

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Abstract

Interleukin 18 (IL-18) is a proinflammatory cytokine that plays a role in host defense by upregulating both innate and acquired immune responses. Analysis of *IL18* polymorphisms may be clinically important since their roles have been recognized in a variety of inflammatory and autoimmune disorders. However, the role of this cytokine polymorphisms in kidney transplant still remains unclear. In this study, we evaluated the associations between *IL18* polymorphisms and graft function assessed by creatinine clearance in kidney transplant recipients. A total of 82 kidney transplant recipients and 183 healthy controls were enrolled, and frequencies of alleles, genotypes and haplotypes for *IL18* polymorphisms were determined and compared with creatinine clearance. The -607C/A (rs1946518) and -137C/G (rs187238) variant alleles in the *IL18* gene were determined by polymerase chain reaction. In our study, no significant association was found between the *IL18* variants and creatinine clearance ($p > 0.05$). Nonetheless, polymorphism analysis revealed an increase in the frequency of the *IL18* major haplotype -607C/-137G in kidney transplant patients (odds ratio 2.57, 95% confidence interval 1.45-4.55, $p = 0.0014$). Finally, we found that *IL18* polymorphisms did not influence the renal function and that *IL18* haplotype -607C/-137G seems to be associated with kidney transplant recipients.

Keywords: IL-18, -607C/A, -137C/G, kidney transplant.

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Introduction

Kidney transplantation is a widely accepted treatment for end-stage renal failure, improving life quality and extending patients survival time. In Brazil, the number of patients on waiting lists for kidney transplantation was about 21,686 in the second semester of 2012, corresponding to 24% of a total of nearly 90,000 patients in dialysis treatment (Solez *et al.*, 2008). Despite all advances in the development of immunosuppressive drugs and the introduction of supportive therapies aimed to decrease acute rejection incidence, several poorly elucidated factors still affect the success of a kidney transplant, leading to a progressive deterioration of the renal function and graft loss.

In synergy with IL-12, IL-18 stimulates the release of IFN-γ by T and B cells, increasing the cytolytic activity of NK cells (Nakamura *et al.*, 1989; Okamura *et al.*, 1995; Micallef *et al.*, 1996; Yoshimoto *et al.*, 1998) and inducing Th1 or Th2 responses, depending on the underlying immunological milieu (Reddy, 2004). IL-18 levels have been reported to be abnormal in some inflammatory and autoimmune diseases (Haas *et al.*, 2005; Novak *et al.*, 2005; Imboden *et al.*, 2006; Thompson and Huphries, 2007). In a murine model of kidney rejection, IL-18 induces several pathways of inflammation when *IL18* and *IL18R* gene expression are upregulated in allografts (Wy-

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burn *et al.*, 2006). Furthermore, increased levels of IL-18 produced by intra-graft macrophages were observed during acute rejection in a rat model (Wyburn *et al.*, 2005). In humans, IL-18 is upregulated in patients during kidney allograft rejection, is expressed in the allograft microenvironment and increased IL-18 serum levels are detected in these patients (Striz *et al.*, 2005). As a corollary, decreased IL-18 levels have been associated with decreased serum creatinine levels in kidney transplant recipients (Parikh *et al.*, 2004).

The *IL18* gene has at least two functional polymorphisms affecting the cytokine production and only few studies have evaluated the association of *IL18* polymorphisms with kidney allotransplantation (Kolesar *et al.*, 2007; Kim *et al.*, 2008; Mittal *et al.*, 2011). In this study, we hypothesized that the genetic variability at the *IL18* gene was associated with kidney allograft function. Considering that creatinine clearance (CrCl) is considered to be a gold standard marker for renal function, we evaluated the association between *IL18* -607C/A (rs1946518) and -137C/G (rs187238) single nucleotide polymorphisms (SNPs) and graft function as assessed by CrCl in kidney transplant recipients.

Material and Methods

Patients and controls

A total of 82 kidney transplant recipients (54 men and 28 women, mean age 38.40 ± 13.21 years) were enrolled from 2010 to 2011 at the Hospital Universitário Onofre Lopes in Natal, in northeastern Brazil. All patients who were attended at the hospital due to disorders in creatinine serum were enrolled, being that 23 of the recruited patients were found in rejection episode during the analysis whereas 59 had no rejection.

Diagnosis of kidney rejection in transplanted patients was realized by histopathological analyses of graft biopsy according to Banff 07 classification (Solez *et al.*, 2008). Biopsies were done on the basis of renal dysfunction, whether worsening or not improving in creatinine levels, *i.e.* no creatinine stabilization. In the absence of biopsy indication, only laboratory tests were collected to monitor the graft function.

As controls of our study, 183 healthy sex- and age-matched individuals (123 men and 60 women, mean age 30.33 ± 8.19 years) were enrolled. These subjects were bone marrow donors at the Hospital Universitário Onofre Lopes and had no known infectious, chronic or autoimmune disorders and neither had kidney diseases and/or undergone transplantation.

This study was approved by the ethical committee of Hospital Universitário Onofre Lopes, Universidade Federal do Rio Grande do Norte, and signed informed consent forms were obtained from all participants.

Creatinine Clearance (CrCl) determination

We evaluated the kidney graft function during a follow-up period of 1 month, 3 months and 6 months after transplantation. The renal function was assessed by determining creatinine clearance levels using the Cockroft-Gault formula calculated as ((140 - age) x weight)/72 x (serum creatinine in mg/dL) x (0.85 if female) (Cockcroft and Gault, 1976).

DNA isolation and amplification of *IL18* promoter polymorphisms

DNA was extracted from peripheral blood leukocytes by Miller's salting out procedure (Miller *et al.*, 1988). *IL18* promoter polymorphisms were evaluated by the polymerase chain reaction (PCR). PCR was done in a final volume of 10 μL containing 100 ng of genomic DNA, 3 pmol of two primers (a common one and the allele-specific one), 2 pmol of each control primer, 0.25 mM dNTP (Pharmacia Biotech, Paris, France), 1.5 mM MgCl2, 0.75 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 1x PCR buffer (0.2 M Tris-HCl, pH 8.5, 0.5 M KCl). The cycling conditions were 3 min at 94 °C, followed by seven cycles of 20 s at 94 °C, 40 s at 64 °C for IL18-607 or 60 s at 68 °C for IL18-137 and 40 s at 72 °C and 25 cycles of 20 s at 94 °C, 40 s at 57 °C for IL18-607 or 20 s at 62 °C for IL18-137 and 40 s at 72 °C, and a final elongation step of 5 min at 72 °C. The amplified products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

Statistical analyses

Comparisons of clinical and descriptive data of the patients were performed with GraphPad Prism 5.0 software, using non-parametric tests, such as the Mann-Whitney test, two-sided Fisher exact test for 2 x 2 contingency tables. An exact test that uses the Metropolis algorithm was done to obtain an unbiased estimate of the exact p-value and its standard error using the RXC software (http://www.marksgeneticsoftware.net/rxc.htm). Allele and genotype frequencies were computed using the direct counting method. Hardy-Weinberg equilibrium was tested by an exact test using GENEPOP 3.4 software (Raymond and Rousset, 1995). The haplotype diversity, as well as the standard derivations, was estimated by the ARLEQUIN 3.11 program (Excoffier and Lischer, 2010). Comparisons of allele, haplotype and genotype frequencies were performed by means of the two-sided Fisher's exact test. In all statistical tests, p-values below 0.05 were considered statistically significant.

Results

Characteristics of the studied population

Demographic, clinical, laboratorial and histopathological features of transplanted patients are shown in Table 1. According to the histopathological analyses, patients

were stratified into rejection and non-rejection groups. To evaluate the differential impact of risk factors on graft function we performed univariate analyses encompassing non-immunological (gender, patient age at transplantation, nephropathy leading to renal failure, type of transplantation, donor age and cold ischemia time) and immunological (an-

Table 1 - Demographic, clinical, laboratorial and histopathological features of kidney transplant patients.

	With rejection n = 23	Without rejection n = 59	p value
Gender			0.1960 (FET)
Male/ Female	18/05	36/23	
Age at transplantation (years)			0.0211 (MW)
Mean	33.09 ± 12.44	40.53 ± 13.01	
Range	27.57-38.601	37.01-44.05	
Nephropathy leading to renal failure			0.8419 (FET)
Hypertension	6	17	
Glomerulosclerosis	0	2	
Glomerulonephritis	5	17	
Other	5	11	
Undetermined	7	12	
Antibody reactivity panel (%)			0.6967 (MW)
0	21	55	
1 to 10	0	0	
10 to 20	0	0	
20 to 30	0	1	
30 to 40	0	1	
40 to 50	1	0	
50 to 60	1	1	
Mean	3.29 ± 7.825	8.29 ± 20.665	
Type of transplantation			0.1499 (FET)
Deceased / living organ donation	13/08	45/12	
Donor age (years)			0.0624 (MW)
Mean	43.75 ± 14.257	33.91 ± 13.83	
Range	36.154 - 51.346	31.701 - 40.117	
Cold ischemia time (h)			0.7623 (MW)
Mean	13.4 ± 6.021	12.93 ± 8.675	
Range	9.093-17.707	9.265-16.593	
Immunossupression			
Mycophenolate	19/1	53/1	0.470 (FET)
Azathioprine	1/19	2/51	1.000 (FET)
Cyclosporine	6/14	11/42	0.535 (FET)
Tacrolimus	14/6	42/12	0.547 (FET)
Sirolimus	0/20	1/53	1.000 (FET)
Prednisone	19/1	50/1	0.487 (FET)
Basiliximab	7/13	16/36	0.782 (FET)
Thymoglobulin	0/21	1/53	1.000 (FET)
HLA-mismatch	0.3 ± 0.6	2.0 ± 1.0	0.126 (MW)
DR HLA-mismatch	0.3 ± 0.6	1.2 ± 0.4	0.214 (MW)
Pre-transplant cross-match			
Negative / positive	20/1	54/0	0.280 (FET)

FET: Fisher exact test. MW: Mann-Whitney test.

tibody reactivity panel, immunosuppressive treatment, HLA-mismatch and pre-transplant cross-match) factors.

Overall, the comparisons between rejection and non-rejection groups revealed non-significant differences, except for the findings that non-rejection patients were older than rejection ones. However, comparisons regarding IL-18 alleles, genotypes and haplotypes between patients with and without transplant rejection showed no statistical differences regarding age at transplantation (data not shown).

Creatinine clearance levels and *IL18* polymorphism

The Creatinine values utilized were reached by the calculation of creatinine clearance (CrCl). The kidney transplant patients showed a progressive increase in renal function during the six months follow up of our study, with the most significant increase occurring in patients who had no rejection. The rejection group showed a discrete renal function improvement as shown in Table 2. The CrCl values when compared with the *IL18* genotypes showed no association.

Table 2 - Kidney function by creatinine clearance (mL/min)[a] of the allograft kidney patients.

	RG (n = 19)	NRG (n = 42)	p value
1 month			
Mean	43.77 ± 23.53	53.35 ± 29.78	0.2589[b]
Range	32.43-55.11	44.07-62.63	
3 months			
Mean	51.53 ± 19.69	66.65 ± 19.32	0.0092[b]
Range	41.74-61.33	59.90-73.39	
6 months			
Mean	52.84 ± 12.26	73.79 ± 20.74	0.0007[b]
Range	45.43-60.25	66.04-81.53	

RG: rejection group; NRG: non-rejection group; [a]estimated glomerular filtration rate calculated with Cockcroft-Gault equation. [b]two-sided Mann-Whitney test.

Genotype and allele frequencies of *IL18* promoter polymorphisms

Genotype distribution of both polymorphisms in healthy controls and patients were in Hardy-Weinberg equilibrium (healthy controls: -607 p = 0.7822, -137 p = 1.0000; Non rejection Group: -607 p = 0.6108, -137 p = 0.6421; Rejection Group: -607 p = 0.0584, -137 p = 0.0831). Allele frequencies and genotype distribution of *IL18* polymorphisms -607A/C and -137C/G in healthy controls and transplant patients are show in Table 3. Haplotype analysis demonstrated an increased frequency of the major haplotype -607C/-137G in kidney transplant patients (odds ratio 2.57, 95% confidence interval 1.45-4.55, p = 0.0014) while the minor haplotype -607A/-137C was more frequent in healthy controls (odds ratio 0.47, 95% confidence interval 0.24-0.92, p = 0.0294) (Table 4).

Histopathological analyses

A total of 49 biopsies were obtained from the 82 kidney transplant recipients. Allograft rejection was present in 23 subjects (28.05%) whereas 26 (31.71%) showed no sign of rejection but presented other histological features of renal transplant pathology, *i.e.* fibrosis, acute tubular necro-

Table 3 - Allele frequencies and genotype distribution (%) of *IL18* polymorphisms -607A/C and -137C/G in healthy controls and transplant patients.

		Patients	Controls	p value[a]	OR	CI95%
-607 A/C						
Alleles	C	88 (0.603)	252 (0.689)	0.0779[a]	0.6884	0.4607 to 1.023
	A	58 (0.397)	114 (0.311)			
Genotype	CC	25 (0.342)	81 (0.443)	0.1262		
	AC	38 (0.521)	90 (0.492)			
	AA	10 (0.137)	12 (0.065)			
Dominant effect	CC *vs.* AC+AA			0.1610 [a]	1.525	0.866 to 2.682
Recessive effect	AA vs.AC+CC			0.0832 [a]	0.4421	0.1820 to 1.074
-137 C/G						
Alleles	G	93 (0.628)	269 (0.735)	0.0189[a]	0.6097	0.4061-0.9154
	C	55 (0.372)	97 (0.265)			
Genotype	GG	25 (0.337)	99 (0.541)	0.0104		
	CG	43 (0.581)	71 (0.388)			
	CC	6 (0.081)	13 (0.071)			
Dominant effect	GG *vs.* CG+CC			0.0037 [a]	2.310	1.316 to 4.055
Recessive effect	CC *vs.* CG+GG			0.7949 [a]	0.8667	0.3164 to 2.374

[a]two-sided Fisher exact test.

Table 4 - Haplotype frequencies (%) of *IL18* promoter in healthy controls and kidney transplant patients.

	Transplant patients	Healthy controls	p value[a]	OR	IC 95%
-607C/ -137G	40 (0.588)	65 (0.357)	0.0014	2.571	1.454 to 4.549
-607C/ -137C	7 (0.103)	19 (0.104)	1	0.9845	0.3941 to 2.459
-607A/ -137G	8 (0.118)	37 (0.203)	0.1401	0.5225	0.2298 to 1.188
-607A /-137C	13 (0.191)	61 (0.335)	0.0294	0.4689	0.2379 to 0.9240

[a]: chi-square test.

sis, nephritis, interstitial inflammation, glomerulonephritis and relapses of the recipient's underlying kidney disease. Fifteen out of 23 patients exhibited acute rejection (18.3%) and 8 exhibited chronic rejection (9.8%). Graft biopsies are not routinely performed in these renal transplanted patients. Prior to biopsy indication, only non-invasive laboratory tests are performed to monitor the graft function. Therefore, this was done only in these 49 patients that presented clinical and/or laboratorial evidence of renal dysfunction.

Discussion

It is well known that IL-18 has a critical role on inflammation and on the immune responses, enhancing IL-12 driven Th1 immune response and, in synergy with IL-4, stimulating the Th2 cell differentiation profile (Nakanishi *et al.*, 2001). The role of IL-18 has recently been demonstrated in a variety of renal disease processes, including ischemia/reperfusion, autoimmune conditions, infections and malignancies (Leslie and Meldrum, 2008). In addition, IL-18 also has an important role on experimental and human renal transplantation (Parikh *et al.*, 2004; Striz *et al.*, 2005; Wyburn *et al.*, 2005, 2006). Therefore, the analysis of *IL18* genetic polymorphisms may improve the understanding of the contribution of this gene to renal transplantation.

The allele variants of the *IL18* gene evaluated in this study may affect protein production, since a change from C to A at position -607 and a change from G to C at position -137 have been associated with differential *IL18* transcription (Giedraitis *et al.*, 2001). Analysis of the *IL18* gene promoter suggested that two single nucleotide variants could cause differences in transcription factor binding. A change from C to A at position -607 disrupts a potential binding site for cAMP-responsive element binding protein. A change at position -137 from G to C changes the H4TF-1 nuclear factor binding site to a binding site for an unknown factor found in the GM-CSF promoter (Giedraitis *et al.*, 2001).

The functional significance of these two SNPs has not been elucidated; however, the -607C and -137G alleles have been associated with higher IL-18 production. In our study, we observed that the frequency of the -607C allele was closely similar in kidney transplant patients and in healthy controls, whereas the -137G allele at single or double doses was underrepresented in renal transplant patients when compared to controls. On the other hand, the -607C/-137G haplotype was overrepresented in kidney transplant patients when compared to controls, whereas the -607A/-137C haplotype was underrepresented in patients, indicating that the ensemble of both polymorphic sites may be important for the control of *IL18* expression.

It is interesting to note that the transcription factors that regulate IL-18 production may differentially act at the promoter region; for instance, the HTF4-1 transcription factor presents a differential action when targeting the -137G/C polymorphic site (Giedraitis *et al.*, 2001). In addition, multiple sclerosis patients exhibiting the -607C/-137G haplotype had higher levels of *IL18* mRNA, while the -607A/-137C haplotype was associated with reduced promoter activity (Giedraitis *et al.*, 2001). Therefore, the study of linkage disequilibrium between -607A/C, -137G/C and eventually other understudied polymorphic sites at the *IL18* promoter region, together with the identification of differentially expressed transcription factors in renal transplanted patients may contribute to the understanding of the role of this gene in transplantation and rejection episodes.

Although we observed no significant differences in terms of *IL18* polymorphic sites when rejection patients were compared to non-rejection ones, and when these polymorphic sites were evaluated according to renal function (ClCr), literature studies reported increased frequency of the -607C allele in Czech kidney transplant patients who presented delayed onset of graft function (Kolesar *et al.*, 2007) and increased frequency of the -137 GG genotype in Korean patients exhibiting acute kidney rejection (Kim *et al.*, 2008).

In our study, we observed that the -137GG genotype was higher in the control group rather than in transplanted patients. However, we strongly believe that this discrepancy regarding -137GG genotype distribution between Kim's study and ours may be due to racial differences in genotype constitution of the two studied population. Furthermore, the frequency of -137 GG, GC and CC genotypes in other Brazilian cohorts of healthy subjects are similar to those shown in our study, suggesting that the distribution of -137GG genotype in our control group is representative of the Brazilian population (Segat *et al.*, 2006, Castelar *et al.*, 2010, Rocha-Júnior *et al.*, 2012). Importantly, this observation gives support to the idea that, although the Brazilian population is genetically heterogeneous when compared to other population, such as the Korean studied by Kim and

collaborators, it seems to be homogeneous when intra-population comparisons are done.

In addition, the combinatorial analysis of alleles at -137 and -607 positions in the context of different clinical conditions, may present different outcomes regarding being a risk factor or presenting a protective function. In support of this hypothesis an increased frequency of -137G/-607A haplotype has been observed in chronic HBV patients (Hirankarn *et al.*, 2007). Similarly, this haplotype was higher in patients with HTLV-1 infection than in healthy subjects, indicating this haplotype as a risk factor for the HTLV-1 infection. On the other hand, protection against HTLV-1 infection was conferred by the -137C/-607C haplotype, since it was increased in healthy controls (Rocha-Júnior *et al.*, 2012). Furthermore, it was shown in studies involving HIV-1 infection that the haplotype -137G/-607C was related to susceptibility to this virus (Segat *et al.*, 2006), and the same genotype was associated with protection against lipodystrophy syndrome development in HIV-1 infection (Castelar *et al.*, 2010).

Ideally, the study of the role of *IL18* promoter region polymorphisms should include the evaluation of the entire promoter segment to unveil the linkage disequilibrium between all polymorphic sites observed in the segment. In addition, the evaluation of IL-18 serum levels, *IL18* expression at the kidney environment, as well as the differential expression profile of the allograft may help to establish relationships among all these factors. In this context, major limitations of this study included the evaluation of only two *IL18* promoter region polymorphic sites and lack of concomitant evaluation of IL-18 levels.

Concluding, although no association between *IL18* polymorphisms with renal function was observed, the increased frequency of the *IL18* -607C/-137G haplotype (containing both alleles associated with high production of IL-18) in kidney recipients deserves further and larger studies to discern whether or not such association may be due to the underlying kidney disorder or to rejection.

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