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Single Nucleotide Polymorphisms of Ubiquitin-Related Genes were Associated with Allograft Fibrosis of Renal Transplant Fibrosis

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Interstitial fibrosis and tubular atrophy (IF/TA) have been recognized as crucial factors contributing to graft loss resulting from chronic renal allograft injuries. Recent studies have indicated a significant association between the progression of organ fibrosis and single nucleotide polymorphisms (SNPs) found on certain genes. Our research sought to understand these potential associations and detect the potential impact of SNPs on ubiquitin-related genes related to allograft fibrosis in kidney transplant recipients.

Material/Methods: There were 200 patients enrolled in this study, from which samples were extracted for total DNA. Targeted next-generation sequencing was used to detect SNPs on 9 genes (*FBXL21, PIAS1/2, SUMO1/2/3/4, UBE2D1,* and *UBE2I*). Minor allele frequency (MAF) and Hardy-Weinberg equilibrium (HWE) tests were used and followed by linkage disequilibrium analysis. General linear models (GLM) were used to identify significant confounding factors. Finally, multiple inheritance models and haplotype analyses were conducted to explore associations between SNPs and the degree of the severity of renal allograft fibrosis.

Results: In total, 144 SNPs were identified in targeted sequencing. After filtering based on results from MAF and HWE tests, 15 tagger SNPs were selected for further analyses of associations. GLMs indicated that the administration of sirolimus significantly contributed to the degree of severity of allograft fibrosis (P=0.011). After adjusting for confounding factors and applying a Bonferroni correction, multiple inheritance model analyses indicated that the recessive model of rs644731 of the *PIAS2* gene was significantly correlated with the occurrence of IF/TA (P=0.01). Furthermore, single-locus based analysis of rs644731 did not indicate that it had a positive influence on IF/TA in a degree-dependent manner. Finally, linkage disequilibrium analysis revealed 3 haplotypes all lacking significant correlation with respect to the IF/TA experimental cohort.

Conclusions: We are the first to reveal that mutations of rs644731 in the *PIAS2* gene were significantly correlated with the progression of IF/TA in kidney transplant recipients.

MeSH Keywords: Kidney Transplantation • Polymorphism, Single Nucleotide • Ubiquitins

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Background

Kidney transplantation is a meritorious treatment of choice for most patients experiencing end-stage renal disease. Although great progress has been made in the effort to use immunosuppressive agents and HLA matching to address renal allograft fibrosis, the onset and progression of this affliction remains a major determining factor which impacts short-term function and long-term outcome for recipients and their allograft status [1]. The pathogenic dynamics of renal allograft fibrosis are driven by a suite of factors including genetic risk, immune system and inflammatory responses, and oxidative stressors, all which may cause subsequent injury of glomeruli, renal interstitium, and tubules [2]. Although previous research has investigated related roles of polymorphisms, accurate genetic *loci* were insufficient to provide a reliable description related to renal fibrosis after transplantation. Furthermore, gene mutations in renal epithelial cells can lead to the activation of epithelial-mesenchymal transition (EMT) inducing renal fibrosis, ultimately indicating that genetic factors are important considerations in the dynamics of pathogenesis of EMT and renal interstitial fibrosis [3].

Since susceptibility genes can provide insights into principal pathological mechanisms, genetic-based analyses of disease can be powerfully insightful for exploring its pathogenesis. The appearance of fibrosis is mainly caused by the emergence of mechanocytes while the degree of fibrosis varies among individuals as a function of polymorphisms within regulatory regions of genes that play roles in transcriptional activation. Thus far, numerous studies have provided support for the importance of the role of single nucleotide polymorphisms (SNPs) in this process, including for coding, noncoding intron, and promoter regions as part of a mix of a larger suite of a number of genes associated with fibrosis disease. It has been reported that rs58542926 on the *TM6SF2* gene is related to hepatic fibrosis [4] and reported that rs738409 on the *PNPLA3* gene is related to liver allograft fibrosis [5]. Furthermore, the chronic renal disease variant rs4730751 is found on the *CAV1* gene and is related to arterial fibrosis [6]. Additionally, the IL-18-607A/C (rs1946518) promoter polymorphism is reported to be correlated with IgA nephropathy and subsequent renal fibrosis [7].

Consistent with these results, a previous study undertaken by several authors of this manuscript determined that tumor necrosis factor (TNF)-α induced EMT via the TNF-α/Akt/Smurf2 signaling pathways [8]. The *Smurf2* gene encodes the E3 ubiquitin-protein ligase Smurf2 in humans, which provides theoretical support for correlations between ubiquitin-related genes and renal fibrosis. Thus, we sought to investigate the association and influences of SNPs with and upon ubiquitin-related genes related to renal allograft interstitial fibrosis. We examined 1 cohort enrolled from our single renal transplant center.

Material and Methods

Ethics statements

Study design, patient enrollment, and procedural protocols were reviewed and approved by the local Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (2016-SR-029). All kidney transplant recipients confirmed their understanding of procedures, protocols, and risks as described and provided through written informed consent. The procedures in our study abided by the ethical standards of the Declarations of Helsinki and Istanbul. All kidney transplant recipients received transplants from donors who had experienced cardiac death

Study design and population

We used a single-center, retrospective, cohort study based approach to explore the influence of SNPs on the transforming growth factor beta (TGFB) signaling pathway and genes (including *FBXL21, PIAS1/2, SUMO1/2/3/4, UBE2D1,* and *UBE2I*) related to the progression of allograft fibrosis in renal transplant recipients. Two hundred kidney transplant recipients, who received renal transplants from February 1, 2015 to September 1, 2018 at the renal transplantation center of the First Affiliated Hospital of Nanjing Medical University, were enrolled in this study. The average follow-up time with patients in our research was 1555±1054 days, and all follow-ups fell between 3 and 5 years after transplantation, and no significant graft failure or decline of renal function was observed. Patients with rejection episodes and delayed graft function (DGF) after transplantation were all eliminated from further inclusion in the study groups. Specifics for inclusion and exclusion criteria were described in methods outlined in greater detail in a previous related study [9].

Clinical data including age, gender, height, and immunosuppressive protocols were independently determined by one of the authors, Zeping Gui.

Investigative biopsies were performed for all transplant recipients enrolled in our study, and histological analyses were conducted by 2 independent nephrologists (Hao Chen and Li Sun) through the use of hematoxylin-eosin (HE), periodic acid Schiff (PAS), Masson, and immunohistological staining following guidelines in Banff 2015 [10]. Allograft fibrosis severity and type/grade were scored using metrics related to the degree of interstitial infiltration and intimal arteritis and by following guidelines in Banff 2015 [10].

Immunosuppressive protocols

Immunosuppressive protocols for all patients included 3 or 4 differently composed treatments of drugs: cyclosporin A or

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tacrolimus, combined with mycophenolate mofetil (MMF) and prednisone, with or without sirolimus which dosage schedules were adjusted according to serum creatinine levels and drug concentrations. Each patient was treated with immunotherapy on the fourth day before, and fourth day after surgery. Detailed information and methodologies for immunosuppressive agent schedules can be found in a previous related study [9].

Sample collection and TS

Detailed information and methodologies for sample collection and TS can be found in a previous related study [9]. Peripheral blood (2 mL) of each patient was used for DNA extraction. We quantitatively analyzed genomic DNA (gDNA) concentration and purity and assessed gene integrity by using agarose gel electrophoresis. We selected from a randomized pool containing upstream and downstream oligonucleotides and gDNA hybrids specific to target regions of interest. We then fragmented gDNA and amplified the adapter-ligated DNA by selective, limited-cycle polymerase chain reaction (PCR). We denatured captured libraries and loaded them into an Illumina cBot instrument following manufacturer protocols. Subsequently, we analyzed sequencing data based around available data for the human reference sequence UCSC hg19 assembly (NCBI build 37.2), using Genome Analysis Tool Kit, Picard Software, and dbSNP 132. We also detected putative somatic variant cells with 2 separate programs: MuTect 1.1.5 and VarScan 2.3.6.

Statistical analysis

Data are presented as mean±standard deviation (SD), except where stated otherwise. We explored minor allele frequency (MAF) and Hardy-Weinberg equilibrium (HWE) by using R package genetics (genetics: Population Genetics, R package version 1.3.8.1.). Linkage disequilibrium (LD) blocks were analyzed using Haploview version 4.2 (Broad Institute, Cambridge, MA, USA). General linear models (GLMs) were used to determine the importance and influence of clinical variables on AR (acute rejection). We used the R Statistics Package SNPassoc (SNPs-based whole genome association studies; R Package Version 1.9-2.) to examine 5 multiple inheritance models based on the different treatments of sirolimus concentrations. These 5 models included: codominant model 1 (major allele homozygotes versus heterozygotes), codominant model 2 (major allele homozygotes versus minor allele homozygotes), dominant model (major allele homozygotes versus minor allele homozygotes plus heterozygotes), recessive model (major allele homozygotes plus heterozygotes versus minor allele homozygotes), overdominant model (heterozygotes versus major allele homozygotes plus minor allele homozygotes), and a log-additive model (major allele homozygotes versus heterozygotes versus minor allele homozygotes). We used chi-square analyses to examine the levels of variance and compare Banff scores when considering 2 or 3 of the selected most important genotypes. All data in our study were analyzed using SPSS Software Version 13.0 (SPSS Inc., Chicago, IL, USA) and $P<0.05$ was considered statistically significant.

Table 1. Basic demographics of patients in this cohort.

Characteristics	Value
Case number	200
Age (years, mean±SD)	44.59±4.09
Gender (Male/Female)	119/81
PRA before renal transplant (%)	0.00
Primary/Secondary renal transplant	200
Primary renal transplant	200
Secondary renal transplant	0
HLA mismatching	4.56±0.34
Type of donor	200
Living-related	24
DCD	176
Administration of sirolimus (%)	12.00
IFTA (n)	69
Mild	32
Moderate	24
Severe	13

SD – standard deviations; PRA – panel reactive antibody; DCD – donor after cardiac death; IFTA – interstitial fibrosis and tubular atrophy.

Results

Patient demographics

A total of 200 patients were enrolled including 119 males, and 81 females. A greater proportion (12%) of patients were treated with sirolimus and none of the panel reactive antibody was found in this cohort before transplantation. Demographics for this patient group are presented in Table 1.

Tagger SNP selection

A total of 15 SNPs were identified. We extracted ubiquitin-related genes and determined the levels of genetic association between 9 associated gene SNPs (*FBXL21, PIAS1/2, SUMO1/2/3/4, UBE2D1,* and *UBE2I*) as well as measures of allograft fibrosis. Our use of reference and alternating alleles helped to support a robust approach and the resultant observational evidence used for genotype analyses (Supplementary Table 1).

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Table 2. Influence of confounding factors on the outcomes of allograft fibrosis by general linear model in this cohort.

Confounding factors	*F* value	*P* value
Gender	0.313	0.7547
Age	0.429	0.6686
Weight	1.175	0.2414
ISD protocol	1.557	0.1210
Duration after renal transplant	0.429	0.6686
Administration of Sirolimus	2.477	0.0134

ISD – immunosuppressive drugs; DGF – delayed graft function.

We defined common variants as those with MAF >0.05, and we set a threshold of 0.05 for HWE values. HWE, MAF, and LD analyses revealed 15 tagger SNPs (rs7283639, rs2838697, rs13050872, rs9306116, rs237025, rs237024, rs237023, rs73288305, rs74377516, rs75362994, rs3737448, rs644731, rs113887072, rs72915074, and rs2066913) that were deemed as statistically frequent SNPs (tSNPs) (MAF >0.05), whereas remainders identified were rare (Supplementary Table 2).

Confounding factor analysis and multiple inheritance model analysis

Based upon relative strength of their association with allograft fibrosis we added markers sequentially as continuous variables to a model using the adjusted confounding factors. In this cohort, confounding factors of patients who were administrated sirolimus were significant (P=0.011) in predicting the incidence of fibrosis, compared with other factors that were not (P>0.05) based upon GLM results. In sum, this suggested that sirolimus had an influence on the outcomes of allograft fibrosis (Table 2).

After applying a Bonferroni correction to adjust the different sirolimus treatments (adjusted P value=0.005) we conducted multiple inheritance model analyses. Synonymous SNP rs644731 was significantly associated with allograft fibrosis [Table 3; odds ratio (OR)=4.42; 95% confidence interval (CI)=1.32–13.64, P=0.01 recessive model; OR=6.57, 95% CI=2.99–14.45, P=1.54 E-07 dominant model; OR=3.5, 95% CI=1.77–6.89, P=0.00017 overdominant model; OR=5.95, 95% CI=2.66–13.3, P=5.08E-07 codominant model; and OR=4.28, 95% CI=2.34–7.83, P=1.73E-07 additive model], suggesting that the risk of allograft fibrosis was strongly correlated with the rs644731 locus, compared with other non-significant tagger SNPs (P>0.005; Supplementary Table 3).

Furthermore, we selected 3 genotypes and compared differences between the degrees of severity of interstitial fibrosis and tubular atrophy (IF/TA) for each of the 3 groups. Results indicated no significant differences (P=0.38) for degrees of IF/TA including mild, moderate, and severe among the CC, CT, and TT genotypes (Table 4). However, we observed that when one T allele appeared (CT genotype), a moderate degree of increase in the severity of IF/TA was noted than in comparison

Table 3. Results of multiple inheritance models in rs644731 adjusted by the administration of sirolimus in 5 models.

rs644731	OR	Lower 95% CI	Upper 95% CI	P value*
Recessive model	4.24	1.32	13.64	0.01
Dominant model	6.57	2.99	14.45	1.54E-07
Overdominant model	3.5	1.77	6.89	0.000169
Codominant model	5.95	2.66	13.3	5.08E-07
Additive model	4.28	2.34	7.83	1.73E-07

OR – odds ratio; CI – confidential interval. * Associations were considered significant if P value is less than 0.005 (Bonferroni corrected-P value).

Table 4. Distributions and analysis of rs644731 in patients with IFTA.

Genotype	IFTA degree			P value
	Mild	Moderate	Severe	
CC	10	5	3	0.38
CT	18	17	6	
TT	4	2	4	

IFTA – interstitial fibrosis and tubular atrophy.

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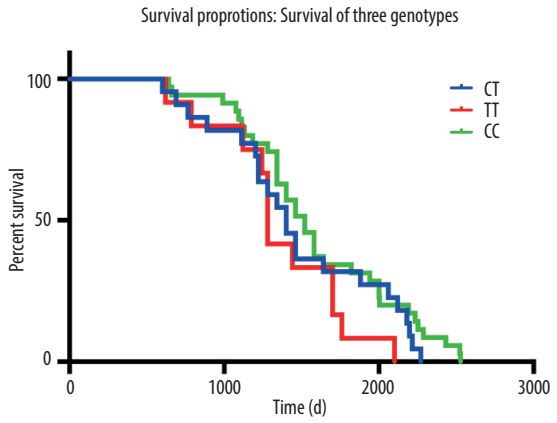

Figure 1. Results of Linkage disequilibrium and haplotypes of all detected single nucleotide polymorphisms.

to the no T allele group. Furthermore, samples with 2 T alleles (TT genotypes) showed a significant increase in the percentage of the degree of severe IF/TA and this was greater than when compared to the other 2 treatments. This result indicated a tendency for the degree of severity of IF/TA to rise with an increase in the number T alleles.

We also performed time-dependent analyses to explore relationships between 3 genotypes and the time from transplantation to biopsy. Results from this survival analysis indicated no significant differences among 3 genotypes which was consistent with previous findings supportive of the idea that duration after renal transplant is not significantly associated with allograft fibrosis (Table 2, Supplementary Table 4).

LD Analysis and haplotype analysis

Haplotype structure and plots of pairwise LD and gene structure are displayed in Figure 1. Each gene was categorized into 3 haplotypes: block 1 (SNPs 4–5: rs7283639, rs2838697), block 2 (SNPs 8-10: rs73288305, rs74377516, rs75362994), and block 3 (SNPs13-14: rs237025, rs237024). Associations between haplotype frequencies are given in Supplementary Table 5.

Haplotype association analyses results using the 3 blocks are listed in Supplementary Table 6. Associations with allograft fibrosis were not statistically significant for block 1 [likelihood ratio (LR)=2.74, *P*=0.74], block 2 (LR=3.46, *P*=0.18), or for block 3 (LR=0.77, *P*=0.68).

Discussion

Previous studies have only focused on a few allograft Ubiquitin-related gene variants, which may be insufficient to capture the full effects of susceptibility related genes. Although such hypotheses have become long-established through randomized trials, evidence for renal allograft fibrosis has been less definite. Our approach was retrospective in targeting the main genes associated with allograft fibrosis. We accordingly used multiple inheritance models and haplotype analyses to test the hypothesis that sigSNP might be an underlying locus related to IF/TA severity after transplantation. We presented the novel finding that the rs644731 variant of the *PIAS2* gene exhibited a statistically significant association with renal allograft fibrosis.

A summary of a genome-wide association study concluded that approximately 80% of trait-associated SNPs are located in non-coding regions [11]. Results from the Encyclopedia of DNA Elements Consortium (ENCODE) attributed important regulatory functions to these noncoding intronic *loci* within the human genome [12]. Rs644731 is an intron of the PIAS2 gene which was found to lack significant linkage disequilibrium. However, rs644731 expression was high for the LD region between an intron SNP (rs737448) and an exon SNP (rs113887072) and might be strongly linked with a potential functional locus exerting a molecular influence on the dynamics of *PIAS2* related gene transcription. Alternative splicing of introns within a gene can act to introduce greater variability in protein sequences translated from a single gene and result in more than just a single unique precursor mRNA transcript with accordingly multiple associated functions. The dynamics of the control of alternative RNA splicing involves a complex network of signaling molecules that respond to a wide range of intracellular and extracellular stimuli [13]. Correspondingly, some introns can enhance expression for the gene containing them through a process known as intron-mediated enhancement (IME) [14]. Further experiments should seek to identify the dynamics of IME that cause a resultant enhancement of expression. So far, one of the most common and best understood mechanisms and approaches is to move the intron upstream from the starting point of transcription, thus removing it and its influence from the final transcript product. If such a change indicates that the intron cannot or has no longer enhanced expression, then inclusion of the intron in the transcript is a vitally important consideration, and the intron may help to induce or solely cause IME [15,16]. Rs644731 is potentially such a type of an intron and may be important in the pathways of regulation of transcription and gene expression, as well as in the sequential steps of the allograft fibrosis pathway. This finding is compatible with the hypothesis that the same locus, such as one intron, can have a similar regulatory type effect on genetic expression. However, we did not find significant differences between the degree of IF/TA (mild, moderate, and severe) and the varied sigSNP genotypes. It is worth noting that with a higher number of T alleles, that the percentage of cases with severe IF/TA displayed an obvious increase. This tendency of increases with T alleles may

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illustrate that the difference might be significant and should be followed up with experimentation using an expanded sample size. Thus, we helped to determine that this locus may represent a potential therapeutic target which may possibly help to reduce the progression of IF/TA in patients after renal transplantations.

Haplotype analyses suggested that the association between locus blocks and allograft fibrosis was not statistically significant. However, our results did indicate a high-risk tendency for patients with renal IF/TA. This possibly indicates that a specific haplotype might be related to higher levels of PIAS2 gene transcription activity. The resultant higher levels of transcription might play an important role in the pathway leading to fibroblast proliferation and the progression of fibrosis. Accordingly, we also investigated other ubiquitin-related genes, but failed to identify an association between gene mutation and the progression of IF/TA. However, results from a previous related study by authors of this manuscript found that having a 1 or 2 copies of the risk allele appeared to significantly increase the ubiquitin-related gene *Smurf2* transcript level in biopsied tissues from the allograft treatment group [17]. Smurf2 mRNA expression increased with the expression of TGF-β1 in the early stages of renal fibrosis and development and it is possible that various methods to induce and enhance Smurf2 expression could be used to some extent in order to slow or prevent the progression of allograft fibrosis [18]. Other ubiquitin-related genes were insignificant in our related analyses which may have resulted because the signal pathways associated with them are yet to be clearly identified. With an increased sample size more potential *loci* might be detected based on an expanded next-generation sequencing approach building from the research we completed herein.

Conclusions from our study while important are still limited in many respects. For example, despite that we adjusted for confounding factors related to the development and progression of allograft fibrosis, sample size was a key factor likely to have impacted our final results and conclusions. Furthermore, current statistically based epidemiological literature does not unanimously concur on when and how to make corrections for confounding factors. Thus, additional studies are needed to clarify roles of genetic polymorphisms on ubiquitin-related genes in renal transplantation patients.

Conclusions

In conclusion, we found that mutations of rs644731 in the *PIAS2* gene were significantly associated with the risk of allograft fibrosis following renal transplantation. Although additional research is needed, our results support the need for a cautious approach as the dynamics of a multifactorial and multigenic disease like allograft fibrosis after renal transplantation are complex and not yet fully understood. Nevertheless, our study provided novel information and a potential new direction for a comprehensive research-based analysis of the importance of and roles that SNPs play in fibrosis-related diseases.

Supplementary Data

Supplementary Table 1. Detailed information of SNP detected in the Ubiquitin-related genes in the cohort.

Chromosome	Location	Reference allele	Alternation allele	Gene name	Function	Gene detail	Avsnp144
chr5	135272403	A	C	FBXL21	Exonic		rs573196792
chr5	135272692	C	T	FBXL21	Intronic		rs150784167
chr5	135272823	G	A	FBXL21	Intronic		rs17702049
chr5	135273078	C	A	FBXL21	Intronic		rs544746984
chr5	135273177	T	C	FBXL21	Exonic		.
chr5	135273298	C	A	FBXL21	Intronic		rs183239904
chr5	135273370	G	T	FBXL21	Intronic		rs10052673
chr5	135276049	G	A	FBXL21	Intronic		rs31549
chr5	135276205	G	C	FBXL21	Exonic		rs76075237
chr5	135276314	C	T	FBXL21	Exonic		rs40986
chr5	135276701	T	C	FBXL21	Intronic		rs31548
chr5	135276814	G	A	FBXL21	Exonic		rs2066913
chr5	135276847	T	C	FBXL21	Exonic		rs31547
chr5	135277204	C	G	FBXL21	Exonic		rs530876112

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Chromosome	Location	Reference allele	Alternation allele	Gene name	Function	Gene detail	Avsnp144
chr15	68346778	T	G	PIAS1	Intronic		.
chr15	68346778	T	C	PIAS1	Intronic		rs537399079
chr15	68348207	C	T	PIAS1	Intronic		.
chr15	68349843	C	G	PIAS1	Intronic		rs182709174
chr15	68349893	A	G	PIAS1	Intronic		rs1489598
chr15	68349918	T	C	PIAS1	Intronic		rs79833223
chr15	68349925	G	A	PIAS1	Intronic		rs183625509
chr15	68378937	G	A	PIAS1	Exonic	.PIAS1: NM_016166: exon2: c.G318A: p.S106S	rs145053928
chr15	68379138	C	T	PIAS1	Intronic		rs186753486
chr15	68434378	C	T	PIAS1	Splicing	NM_016166: exon3: c.554+10C>T.	rs750502048
chr15	68434379	G	C	PIAS1	Intronic		rs117588299
chr15	68434428	A	G	PIAS1	Intronic		.
chr15	68434744	G	A	PIAS1	Intronic		rs145280358
chr15	68439101	A	G	PIAS1	Intronic		rs576273919
chr15	68445850	C	G	PIAS1	Intronic		.
chr15	68445912	T	A	PIAS1	Intronic		rs11633620
chr15	68468049	C	T	PIAS1	Exonic	.PIAS1: NM_016166: exon10: c.C1244T: p.P415L	.
chr15	68468098	A	G	PIAS1	Exonic	.PIAS1: NM_016166: exon10: c.A1293G: p.G431G	rs113555272
chr15	68468136	C	T	PIAS1	Intronic		.
chr15	68468695	T	C	PIAS1	Intronic		rs12438361
chr15	68473443	T	C	PIAS1	Intronic		rs3759823
chr15	68473808	G	A	PIAS1	Intronic		rs75673552
chr15	68476069	A	G	PIAS1	Intronic		.
chr15	68479858	G	C	PIAS1	Intronic		.
chr15	68480086	A	G	PIAS1	Exonic	.PIAS1: NM_016166: exon14: c.A1869G: p.E623E	rs191408288
chr15	68480226	A	G	PIAS1	Utr3	NM_016166: c.*53A>G.	rs372957210
chr18	44395368	T	C	PIAS2	Intronic		rs3737448
chr18	44398111	C	T	PIAS2	Utr3	NM_173206: c.*229G>A.	rs10502879
chr18	44398183	A	T	PIAS2	Utr3	NM_173206: c.*157T>A.	.
chr18	44398464	G	A	PIAS2	Intronic		rs149740503
chr18	44398557	A	T	PIAS2	Intronic		rs183321210
chr18	44401052	G	A	PIAS2	Intronic		rs146442641
chr18	44407993	G	A	PIAS2	Exonic	.PIAS2: NM_004671: exon11: c.C1437T: p.D479D,PIAS2: NM_173206: exon11: c.C1437T: p.D479D	rs35451178
chr18	44409581	C	A	PIAS2	Intronic		rs72907142
chr18	44409617	G	A	PIAS2	Intronic		.
chr18	44416287	G	A	PIAS2	Intronic		.

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Chromosome	Location	Reference allele	Alternation allele	Gene name	Function	Gene detail	Avsnp144
chr18	44416608	C	T	PIAS2	Intronic		rs72907148
chr18	44423925	A	G	PIAS2	Intronic		rs77040088
chr18	44424122	A	G	PIAS2	Intronic		rs188584114
chr18	44424939	G	A	PIAS2	Intronic		rs372024400
chr18	44424995	A	G	PIAS2	Intronic		rs150885589
chr18	44426800	T	C	PIAS2	Exonic	.PIAS2: NM_004671: exon6: c.A731G: p.Y244C,PIAS2: NM_173206: exon6: c.A731G: p.Y244C	rs114135676
chr18	44426877	C	T	PIAS2	Intronic		rs644731
chr18	44435407	A	G	PIAS2	Splicing	NM_004671: exon6: c.636-9T>C;NM_173206: exon6: c.636-9T>C.	rs764656048
chr18	44470481	T	C	PIAS2	Intronic		rs539782995
chr18	44470706	G	A	PIAS2	Exonic	.PIAS2: NM_004671: exon2: c.C336T: p.H112H,PIAS2: NM_173206: exon2: c.C336T: p.H112H	rs113887072
chr18	44470825	A	G	PIAS2	Exonic	.PIAS2: NM_004671: exon2: c.T217C: p.S73P,PIAS2: NM_173206: exon2: c.T217C: p.S73P	.
chr18	44483961	T	C	PIAS2	Intronic		rs72915074
chr18	44496847	C	T	PIAS2	Intronic		rs567309045
chr18	44497099	C	A	PIAS2	Intronic		rs530227612
chr18	44497124	C	T	PIAS2	Intronic		.
chr18	44497173	C	A	PIAS2	Intronic		.
chr18	44497338	T	G	PIAS2	Utr5	NM_004671: c.-30A>C;NM_173206: c.-30A>C.	.
chr2	203072108	G	A	SUMO1	Intronic		.
chr2	203072889	T	C	SUMO1	Intronic		.
chr2	203079307	T	C	SUMO1	Intronic		rs3769817
chr2	203096545	C	A	SUMO1	Intronic		rs116081766
chr2	203103241	G	T	SUMO1	Utr5	NM_001005781: c.-67C>A;NM_001005782: c.-67C>A;NM_003352: c.-67C>A.	.
chr17	73177444	C	A	SUMO2	Intronic		rs1471453
chr17	73179065	C	G	SUMO2	Utr5	NM_001005849: c.-136G>C;NM_006937: c.-136G>C.	.
chr21	46226786	A	G	SUMO3	Utr3	NM_001286416: c.*80T>C;NM_006936: c.*80T>C.	rs1051331
chr21	46227163	A	G	SUMO3	Intronic		rs2329902
chr21	46227968	T	G	SUMO3	Intronic		.
chr21	46228150	G	A	SUMO3	Intronic		.
chr21	46228153	C	T	SUMO3	Intronic		.
chr21	46228154	G	A	SUMO3	Intronic		rs752652207
chr21	46228155	C	T	SUMO3	intronic		rs757331290

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Chromosome	Location	Reference allele	Alternation allele	Gene name	Function	Gene detail	Avsnp144
chr21	46228157	T	G	SUMO3	Intronic		rs765382230
chr21	46228165	T	C	SUMO3	Intronic		rs7283639
chr21	46228170	T	G	SUMO3	Intronic		rs188978703
chr21	46228243	T	C	SUMO3	Intronic		rs141141907
chr21	46228662	C	T	SUMO3	Intronic		.
chr21	46228930	C	T	SUMO3	Intronic		rs235293
chr21	46228945	C	T	SUMO3	Intronic		rs564735586
chr21	46228949	G	C	SUMO3	Intronic		.
chr21	46233836	C	A	SUMO3	Exonic	.SUMO3: NM_001286416: exon2: c.G205T: p.V69F	rs2838697
chr21	46233863	G	C	SUMO3	Exonic	.SUMO3: NM_001286416: exon2: c.C178G: p.L60V	rs13050872
chr21	46233866	T	C	SUMO3	Exonic	.SUMO3: NM_001286416: exon2: c.A175G: p.S59G	rs9981327
chr21	46234079	T	A	SUMO3	Intronic	..	rs9306116
chr6	149721690	G	A	SUMO4	Exonic	.SUMO4: NM_001002255: exon1: c.G163A: p.V55M	rs237025
chr6	149721778	T	C	SUMO4	Exonic	.SUMO4: NM_001002255: exon1: c.T251C: p.I84T	rs777445425
chr6	149721800	G	A	SUMO4	Exonic	.SUMO4: NM_001002255: exon1: c.G273A: p.T91T	rs145312495
chr6	149721965	T	C	SUMO4	Utr3	NM_001002255: c.*150T>C.	rs237024
chr6	149722040	A	G	SUMO4	Utr3	NM_001002255: c.*225A>G.	rs237023
chr10	60095105	G	T	UBE2D1	Intronic		rs112660736
chr10	60121139	A	G	UBE2D1	Exonic	.UBE2D1: NM_003338: exon2: c.A66G: p.S22S	rs759280904
chr10	60121240	C	T	UBE2D1	Intronic		.
chr10	60123486	A	G	UBE2D1	Intronic		rs73288305
chr10	60123523	A	G	UBE2D1	Intronic		.
chr10	60124703	C	T	UBE2D1	Intronic		.
chr10	60127627	A	G	UBE2D1	Intronic		rs531786752
chr10	60127639	G	T	UBE2D1	Intronic		rs74377516
chr10	60127798	T	C	UBE2D1	Intronic		.
chr10	60127838	G	A	UBE2D1	Intronic		rs75362994
chr10	60128364	T	C	UBE2D1	Intronic		rs3802699
chr10	60128583	A	G	UBE2D1	Utr3	NM_001204880: c.*58A>G;NM_003338: c.*58A>G.	rs148198083
chr16	1363878	C	T	UBE2I	Intronic		rs9926183
chr16	1363927	A	G	UBE2I	Intronic		.
chr16	1364140	T	C	UBE2I	Intronic		rs9941160
chr16	1364158	G	A	UBE2I	Intronic		.
chr16	1364251	C	T	UBE2I	Intronic		rs201695180
chr16	1364281	T	C	UBE2I	Intronic		rs4984806

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Chromosome	Location	Reference allele	Alternation allele	Gene name	Function	Gene detail	Avsnp144
chr16	1364365	A	G	UBE2I	Exonic	.UBE2I: NM_003345: exon3: c.A138G: p.P46P,UBE2I: NM_194260: exon3: c.A138G: p.P46P,UBE2I: NM_194261: exon3: c.A138G: p.P46P,UBE2I: NM_194259: exon4: c.A138G: p.P46P	rs4610
chr16	1365612	G	A	UBE2I	Intronic		rs781398317
chr16	1365915	C	T	UBE2I	Intronic		rs112302601
chr16	1365935	C	G	UBE2I	Intronic		rs4984807
chr16	1365943	C	T	UBE2I	Intronic		rs7186045
chr16	1365967	C	T	UBE2I	Intronic		rs4984808
chr16	1369612	C	T	UBE2I	Intronic		rs201661304
chr16	1369730	A	G	UBE2I	Intronic		rs9933497
chr16	1369837	C	T	UBE2I	Intronic		.
chr16	1369926	T	C	UBE2I	Intronic		rs909915
chr16	1370203	G	A	UBE2I	Exonic	.UBE2I: NM_003345: exon5: c.G252A: p.P84P,UBE2I: NM_194260: exon5: c.G252A: p.P84P,UBE2I: NM_194261: exon5: c.G252A: p.P84P,UBE2I: NM_194259: exon6: c.G252A: p.P84P	rs758216436
chr16	1370303	T	C	UBE2I	Intronic		rs909916
chr16	1370309	C	G	UBE2I	Intronic		rs909917
chr16	1370383	C	T	UBE2I	Intronic		rs148789348
chr16	1370575	G	C	UBE2I	Intronic		.
chr16	1370597	G	C	UBE2I	Intronic		rs4017786
chr16	1370614	C	G	UBE2I	Intronic		rs8063770
chr16	1370630	A	G	UBE2I	Intronic		rs8043720
chr16	1370682	G	A	UBE2I	Intronic		rs79005361
chr16	1370698	G	A	UBE2I	Intronic		rs571836605
chr16	1370716	C	T	UBE2I	Intronic		rs142273742
chr16	1370729	C	A	UBE2I	Intronic		rs909918
chr16	1374513	G	A	UBE2I	Intronic		rs2369700
chr16	1374524	A	G	UBE2I	Intronic		rs761059
chr16	1374629	G	T	UBE2I	Intronic		.
chr16	1374656	A	G	UBE2I	Intronic		rs761060
chr16	1374785	G	A	UBE2I	Exonic	.UBE2I: NM_003345: exon7: c.G468A: p.A156A,UBE2I: NM_194260: exon7: c.G468A: p.A156A,UBE2I: NM_194261: exon7: c.G468A: p.A156A,UBE2I: NM_194259: exon8: c.G468A: p.A156A	rs762904858
chr16	1374818	A	G	UBE2I	UTR3	NM_003345: c.*24A>G;NM_194259: c.*24A>G;NM_194260: c.*24A>G;NM_194261: c.*24A>G.	rs8063

SNP – single nuclear polymorphism.

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Supplementary Table 2. Outcomes of HWE and MAF calculation for all detected SNPs in the cohort.

Gene name	SNP	Location	MAF	HWE
SUMO1	.	203072108	0.0025	1.00
SUMO1	.	203072889	0.005	1.00
SUMO1	rs3769817	203079307	0.1575	0.00
SUMO1	rs116081766	203096545	0.0025	1.00
SUMO1	.	203103241	0.0025	1.00
SUMO2	rs1471453	73177444	0.05	0.00
SUMO2	.	73179065	0.0025	1.00
SUMO3	rs1051331	46226786	0.0025	1.00
SUMO3	rs2329902	46227163	0.13	0.00
SUMO3	.	46227968	0.0025	1.00
SUMO3	.	46228150	0.0025	1.00
SUMO3	.	46228153	0.0025	1.00
SUMO3	rs752652207	46228154	0.0025	1.00
SUMO3	rs757331290	46228155	0.0025	1.00
SUMO3	rs765382230	46228157	0.005	1.00
SUMO3	rs7283639	46228165	0.155	0.27
SUMO3	rs188978703	46228170	0.02	0.07
SUMO3	rs141141907	46228243	0.0025	1.00
SUMO3	.	46228662	0.0025	1.00
SUMO3	rs235293	46228930	0.0125	1.00
SUMO3	rs564735586	46228945	0.0025	1.00
SUMO3	.	46228949	0.0025	1.00
SUMO3	rs2838697	46233836	0.445	0.48
SUMO3	rs13050872	46233863	0.085	0.15
SUMO3	rs9981327	46233866	0.0025	1.00
SUMO3	rs9306116	46234079	0.4375	0.67
SUMO4	rs237025	149721690	0.305	0.32
SUMO4	rs777445425	149721778	0.0025	1.00
SUMO4	rs145312495	149721800	0.0025	1.00
SUMO4	rs237024	149721965	0.305	0.32
SUMO4	rs237023	149722040	1	NA
UBE2D1	rs112660736	60095105	0.015	0.04
UBE2D1	rs759280904	60121139	0.0025	1.00
UBE2D1	.	60121240	0.0025	1.00
UBE2D1	rs73288305	60123486	0.3025	0.24
UBE2D1	.	60123523	0.0025	1.00
UBE2D1	.	60124703	0.0025	1.00
UBE2D1	rs531786752	60127627	0.0025	1.00
UBE2D1	rs74377516	60127639	0.2975	0.17
UBE2D1	.	60127798	0.0025	1.00
UBE2D1	rs75362994	60127838	0.3025	0.24

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Gene name	SNP	Location	MAF	HWE
UBE2D1	rs3802699	60128364	0.0025	1.00
UBE2D1	rs148198083	60128583	0.0475	1.00
UBE2I	rs9926183	1363878	0.035	0.00
UBE2I	.	1363927	0.0025	1.00
UBE2I	rs9941160	1364140	1	NA
UBE2I	.	1364158	0.0025	1.00
UBE2I	rs201695180	1364251	0.0025	1.00
UBE2I	rs4984806	1364281	1	NA
UBE2I	rs4610	1364365	0.0375	0.02
UBE2I	rs781398317	1365612	0.0025	1.00
UBE2I	rs112302601	1365915	0.0025	1.00
UBE2I	rs4984807	1365935	0.395	0.00
UBE2I	rs7186045	1365943	0.015	1.00
UBE2I	rs4984808	1365967	0.0725	0.00
UBE2I	rs201661304	1369612	0.0075	1.00
UBE2I	rs9933497	1369730	1	NA
UBE2I	.	1369837	0.0025	1.00
UBE2I	rs909915	1369926	0.185	0.00
UBE2I	rs758216436	1370203	0.0025	1.00
UBE2I	rs909916	1370303	1	NA
UBE2I	rs909917	1370309	1	NA
UBE2I	rs148789348	1370383	0.0125	1.00
UBE2I	.	1370575	0.0025	1.00
UBE2I	rs4017786	1370597	1	NA
UBE2I	rs8063770	1370614	1	NA
UBE2I	rs8043720	1370630	1	NA
UBE2I	rs79005361	1370682	0.0475	0.36
UBE2I	rs571836605	1370698	0.0025	1.00
UBE2I	rs142273742	1370716	0.0075	0.01
UBE2I	rs909918	1370729	1	NA
UBE2I	rs2369700	1374513	0.005	0.00
UBE2I	rs761059	1374524	0.0175	0.00
UBE2I	.	1374629	0.0025	1.00
UBE2I	rs761060	1374656	0.0425	0.00
UBE2I	rs762904858	1374785	0.0025	1.00
UBE2I	rs8063	1374818	0.0475	0.01
PIAS1	.	68346778	0.0025	1.00
PIAS1	rs537399079	68346778	0.0025	1.00
PIAS1	.	68348207	0.0025	1.00
PIAS1	rs182709174	68349843	0.0025	1.00
PIAS1	rs1489598	68349893	0.165	0.12
PIAS1	rs79833223	68349918	0.0325	1.00
PIAS1	rs183625509	68349925	0.01	1.00

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Gene name	SNP	Location	MAF	HWE
PIAS1	rs145053928	68378937	0.0125	1.00
PIAS1	rs186753486	68379138	0.01	1.00
PIAS1	rs750502048	68434378	0.0025	1.00
PIAS1	rs117588299	68434379	0.0225	1.00
PIAS1	.	68434428	0.0025	1.00
PIAS1	rs145280358	68434744	0.01	1.00
PIAS1	rs576273919	68439101	0.0025	1.00
PIAS1	.	68445850	0.0025	1.00
PIAS1	rs11633620	68445912	1	NA
PIAS1	.	68468049	0.0025	1.00
PIAS1	rs113555272	68468098	0.0225	1.00
PIAS1	.	68468136	0.0025	1.00
PIAS1	rs12438361	68468695	0.0375	0.00
PIAS1	rs3759823	68473443	0.0225	1.00
PIAS1	rs75673552	68473808	0.0025	1.00
PIAS1	.	68476069	0.0025	1.00
PIAS1	.	68479858	0.0025	1.00
PIAS1	rs191408288	68480086	0.01	1.00
PIAS1	rs372957210	68480226	0.01	1.00
PIAS2	rs3737448	44395368	0.0925	0.68
PIAS2	rs10502879	44398111	0.0025	1.00
PIAS2	.	44398183	0.0025	1.00
PIAS2	rs149740503	44398464	0.0025	1.00
PIAS2	rs183321210	44398557	0.005	1.00
PIAS2	rs146442641	44401052	0.015	1.00
PIAS2	rs35451178	44407993	0.0025	1.00
PIAS2	rs72907142	44409581	0.005	1.00
PIAS2	.	44409617	0.0025	1.00
PIAS2	.	44416287	0.0025	1.00
PIAS2	rs72907148	44416608	0.005	1.00
PIAS2	rs77040088	44423925	0.01	1.00
PIAS2	rs188584114	44424122	0.0025	1.00
PIAS2	rs372024400	44424939	0.0025	1.00
PIAS2	rs150885589	44424995	0.0025	1.00
PIAS2	rs114135676	44426800	0.005	1.00
PIAS2	rs644731	44426877	0.3025	0.32
PIAS2	rs764656048	44435407	0.0025	1.00
PIAS2	rs539782995	44470481	0.0025	1.00
PIAS2	rs113887072	44470706	0.0575	0.49
PIAS2	.	44470825	0.0025	1.00
PIAS2	rs72915074	44483961	0.005	1.00
PIAS2	rs567309045	44496847	0.0025	1.00
PIAS2	rs530227612	44497099	0.0025	1.00

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Gene name	SNP	Location	MAF	HWE
PIAS2	.	44497124	0.0025	1.00
PIAS2	.	44497173	0.0025	1.00
PIAS2	.	44497338	0.0025	1.00
FBXL21	rs573196792	135272403	0.0025	1.00
FBXL21	rs150784167	135272692	0.0025	1.00
FBXL21	rs17702049	135272823	0.01	1.00
FBXL21	rs544746984	135273078	0.0025	1.00
FBXL21	.	135273177	0.0025	1.00
FBXL21	rs183239904	135273298	0.005	1.00
FBXL21	rs10052673	135273370	0.0175	1.00
FBXL21	rs31549	135276049	0.1125	0.00
FBXL21	rs76075237	135276205	0.0025	1.00
FBXL21	rs40986	135276314	0.22	0.04
FBXL21	rs31548	135276701	0.22	0.04
FBXL21	rs2066913	135276814	0.225	0.84
FBXL21	rs31547	135276847	0.22	0.04
FBXL21	rs530876112	135277204	0.005	1.00

SNP – single nuclear polymorphism; MAF – minor allele frequency; HWE – Hardy Weinberg equilibrium; NA – not available.

Supplementary Table 3. Results of logistic regression adjusted by the administration of sirolimus in non-significant tagger SNPs by five models.

SNPs	OR	Lower 95% CI	Upper 95% CI	P value
Recessive model				
rs644731	4.24	1.32	13.64	0.01
rs3737448	NA	0	NA	0.08
rs75362994	0.53	0.17	1.6	0.24
rs7283639	1.95	0.42	9.03	0.40
rs113887072	0	0	NA	0.41
rs1489598	2.55	0.16	41.59	0.52
rs13050872	0.5	0.03	8.36	0.62
rs2066913	0.7	0.14	3.44	0.65
rs2838697	0.93	0.43	2.01	0.85
Dominant model				
rs2838697	0.62	0.32	1.19	0.15
rs2066913	0.65	0.34	1.24	0.18
rs3737448	1.66	0.75	3.64	0.21
rs237025	0.74	0.4	1.38	0.34
rs113887072	0.61	0.21	1.79	0.36
rs75362994	1.32	0.71	2.47	0.38
rs7283639	1.18	0.59	2.35	0.64
rs13050872	0.85	0.35	2.04	0.71
rs1489598	0.92	0.47	1.79	0.80

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SNPs	OR	Lower 95% CI	Upper 95% CI	P value
Overdominant model				
rs75362994	1.7	0.9	3.21	0.10
rs2838697	0.68	0.36	1.28	0.23
rs2066913	0.68	0.35	1.31	0.24
rs237025	0.72	0.38	1.35	0.30
rs3737448	1.45	0.64	3.25	0.38
rs113887072	0.65	0.22	1.93	0.43
rs1489598	0.87	0.44	1.72	0.69
rs13050872	0.9	0.37	2.24	0.83
rs7283639	1.04	0.51	2.16	0.91
Codominant model				
rs3737448	1.49	0.66	3.34	0.14
rs75362994	1.58	0.82	3.05	0.20
rs2838697	0.59	0.29	1.19	0.33
rs2066913	0.65	0.33	1.28	0.41
rs113887072	0.65	0.22	1.92	0.52
rs237025	0.71	0.37	1.36	0.58
rs7283639	1.08	0.52	2.25	0.69
rs1489598	0.88	0.45	1.75	0.76
rs13050872	0.89	0.36	2.22	0.86
Additive model				
rs3737448	1.77	0.84	3.69	0.13
rs2066913	0.7	0.4	1.22	0.20
rs2838697	0.79	0.51	1.23	0.30
rs113887072	0.6	0.21	1.69	0.31
rs237025	0.83	0.5	1.38	0.48
rs7283639	1.22	0.69	2.15	0.49
rs13050872	0.83	0.38	1.81	0.64
rs75362994	1.03	0.65	1.63	0.90
rs1489598	0.97	0.51	1.83	0.92

SNP – single nuclear polymorphisms; OR – odds ratio; CI – confidential interval; NA – not available.

Supplementary Table 4. Results of survival analysis between three genotypes and the time from transplantation to biopsy in this cohort.

Confounding factors	P value
Three genotypes (CT *vs.* TT *vs.* CC)	0.1573
CT *vs.* TT	0.2586
CT *vs.* CC	0.2833
CC *vs.* TT	0.0864

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Supplementary Table 5. Results of linkage disequilibrium haplotype analysis in this cohort.

Block 1	rs7283639	rs2838697	Haplotype frequency (%)
H1	G	A	55.5
H2	G	C	29
H3	C	C	15.5
Block 2	rs73288305	rs74377516	rs75362994	Haplotype frequency (%)
H1	A	T	T	69.7
H2	T	G	A	29.8
Block 3	rs237025	rs237024	Haplotype frequency (%)
H1	A	C	69.5
H2	T	G	30.5

Supplementary Table 6. Results of haplotype analysis among detected blocks in this cohort.

Blocks	Likelihood ratio	Test df	P value
Block 1	2.74	5	0.74
Block 2	3.46	2	0.18
Block 3	0.77	2	0.68

df – degree of freedom.

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