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Acute rejection in kidney transplantation and the evaluation of associated polymorphisms (SNPs): the importance of sample size

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Abstract

Background: Acute rejection (AR) is one of the most frequent complications after kidney transplantation (KT). Scientific evidence reports that some single-nucleotide polymorphisms (SNPs) located in genes involved in the immune response and in the pharmacokinetics and pharmacodynamics of immunosuppressive drugs are associated with rejection in renal transplant patients. The aim of this study was to evaluate some SNPs located in six genes: interleukin-10 (*IL-10*), tumor necrosis factor (*TNF*), adenosine triphosphate-binding cassette sub-family B member 1 (*ABCB1*), uridine diphosphate glucuronosyltransferase family 1 member A9 (*UGT1A9*), inosine monophosphate dehydrogenase 1 (*IMPDH1*) and *IMPDH2*.
Methods: We enrolled cases with at least one AR after KT and two groups of controls: patients without any AR after KT and healthy blood donors. Genetic analysis on DNA was performed. The heterozygosity (HET) was determined and the Hardy-Weinberg equilibrium (HWE) test was performed for each SNP. The sample size was calculated using the QUANTO program and the genetic associations were calculated using the SAS program (SAS Institute Inc., Cary, NC, USA).
Results: In our previous preliminary study (sample size was not reached for cases), the results showed that patients with the C allele in the SNP rs1045642 and the A allele in the SNP rs2032582 of the *ABCB1* gene had more frequent AR. In contrast, with the achievement of sample size, the trend of the previous data was not confirmed.
Conclusions: Our study highlights a fundamental aspect of scientific research that is generally presumed, i.e. the sample size of groups enrolled for a scientific study. We believe that our study will make a significant contribution to the scientific community in the discussion of the importance of the analysis and the achievement of sample size to evaluate the associations between SNPs and the studied event.

Keywords: acute rejection; kidney transplantation; sample size; single-nucleotide polymorphisms (SNPs).

Introduction

Patients with end-stage renal disease may require kidney transplantation (KT) as renal replacement therapy [1–3].

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Acute rejection (AR) is one of the most frequent complications after transplantation. It is an important cause of graft loss that may occur at any time point in the lifetime of renal transplant recipients, independent of age and gender [1, 4], especially if there is a change in the immunosuppressive therapy or in the case of infections. It is suspected every time there is an acute graft dysfunction, usually measured by a rapid increase in serum creatinine [5]. The diagnosis is generally difficult, and it is based on the exclusion of other causes of graft dysfunction. However, renal biopsy is still a useful instrument for diagnosis, establishment of prognosis and treatment [5].

There is a great variability in response to drugs among individuals, due to environmental and physiopathological factors, which may modify the bioavailability and the kinetics of drugs. Particularly, in the recent years, it was observed how the inter-individual variability in response to drugs is caused by polymorphic variants located in genes codifying for cellular receptors, carriers and transducers [6] that are targets of the same drugs [7].

The outcome of KT highlights that there is a substantial variability in response to the same immunosuppressive treatment: genetic differences among the individuals may influence graft [7] and patient survival [8].

Scientific evidence reports that some single-nucleotide polymorphisms (SNPs) located in genes involved in the immune response and in the pharmacokinetics and pharmacodynamics of immunosuppressive drugs are associated with rejection in renal transplant patients [9, 10]. Moreover, the role of cytokines and other mediators of immune response involved in the development of the rejection required an even more specific investigation of polymorphisms and the expression of various genes [11, 12].

In this study, we selected and analyzed 12 SNPs located in six genes that are targets of the immune response and of the immunosuppressive therapy of renal transplant patients. Interleukin-10 (*IL-10*) and tumor necrosis factor (*TNF*) are targets of the immune response; adenosine triphosphate-binding cassette sub-family B member 1 (*ABCB1*) is connected with the pharmacokinetics of tacrolimus; uridine diphosphate glucuronosyltransferase family 1 member A9 (*UGT1A9*) influences the bioavailability of mycophenolic acid (MPA); inosine monophosphate dehydrogenase 1 (*IMPDH1*) and *IMPDH2* are targets of MPA.

The aim of our study was to determine the possible genetic associations between the SNPs and the AR event in KT, and to identify a specific allele associated with AR. The ultimate goal is to improve the identification of individuals at higher risk of AR, optimally tailoring their immunosuppressive therapy.

Materials and methods

Enrollment

This is an observational, non-matched, case-control study. The study protocol was approved by the Vicenza Ethics Committee of San Bortolo Hospital n° 32/11, and by the Ethics Committee of the Udine University Hospital n° 42/16.

For the realization of this study, we enrolled three groups of individuals: case group (patients with at least one AR event after KT, confirmed by histological examination with renal biopsy after seeing a fast increase in serum creatinine levels); control I group (patients without any AR episode after KT); and control II group (healthy blood donors).

All the involved individuals were Caucasians aged more than 18 years, and all had signed the informed consent of participation in the study, as recommended by the Declaration of Helsinki.

A total number of 50 patients of the case group were enrolled at the Transplant Centre of the Department of Nephrology, Dialysis and Transplantation of San Bortolo Hospital, Vicenza, and 24 were enrolled at the Transplant Centre of the Department of Nephrology of the Udine University Hospital, Udine; all patients enrolled in the two centers received the same treatments. All patients of the control I group were enrolled at the Transplant Centre of the Department of Nephrology, Dialysis and Transplantation of San Bortolo Hospital, Vicenza. The individuals of the control II group were enrolled at San Bortolo Hospital, Vicenza.

Sample collection

For all patients of the case and control I groups, we collected two peripheral blood samples [9 mL in ethylenediaminetetraacetic acid (EDTA) tubes] after a follow-up outpatient medical examination and the signature of the informed consent. For the control II group, we collected the blood sample in an anonymous way.

All blood samples were processed in the International Renal Research Institute Vicenza (IRRIV) Laboratories of the Department of Nephrology, Dialysis and Transplantation, San Bortolo Hospital Vicenza.

SNP selection in target genes

We studied 12 SNPs located in six different genes that are targets of the immune response and of the immunosuppressive therapy in renal transplant patients: rs1800872 in the *IL-10* gene; rs1800629 in the *TNF* gene; rs1045642, rs1128503 and rs2032582 in the *ABCB1* gene; rs6714486, rs2741045, rs2741046 and rs17868320 in the *UGT1A9* gene; rs11706052 in the *IMPDH2* gene; and rs2278293 and rs2278294 in the *IMPDH1* gene.

These SNPs were selected based on the previously published literature [10, 13].

Genetic analysis

The protocol performed for the study is comprehensive of DNA extraction and purification, DNA quantitative and qualitative evaluation,

polymerase chain reaction (PCR), agarose gel electrophoresis, fragment purification, Sanger sequencing reaction, purification of the sequences and capillary electrophoresis.

DNA was extracted from each blood sample using the NorDiag Arrow instrument with the Blood DNA 200 Extraction Kit disposable cartridge (DiaSorin Ireland Ltd., Dublin, Ireland). The instrument uses a magnetic bead-based extraction method.

The NanoPhotometer N50 Touch (Implen, Schatzbogen, Germany) instrument, based on a spectrophotometric technology, was used for DNA qualitative and quantitative evaluation.

Before preparing the reaction mix for the PCR, we adjusted the necessary parameters: primer design and annealing temperature (Ta). The primers were designed using the Primer3 program (Whitehead Institute, Cambridge, MA, USA), as shown in Table 1.

To perform the PCR, we used the standardized reaction mix AmpliTaq Gold 360 MasterMix (Applied Biosystems, Foster City, CA, USA), which contains a specific Hot Start Taq polymerase enzyme. The protocol (thermal cycling) was as follows: initial denaturation: 10 min at 95°C; 35 amplification cycles of: denaturation: 30 s at 95°C, annealing: according to the SNPs, extension: 40 s at 72°C; final extension cycle: 10 min at 72°C.

The PCR products were verified by electrophoresis in 2% agarose gel (Starpure Agarose melted in Tris Borate EDTA buffer) stained with Midori Green Direct (Nippon Genetics, Dueren, Germany).

Purification of the fragments was performed using Illustra ExoProStar 1-Step (GE Healthcare Bio-Sciences, Pittsburgh, MA, USA), according to the instructions of the manufacturer.

The Sanger sequencing reaction was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the instructions of the manufacturer. The products of the reaction were purified using Centri-Sep Columns (Princeton Separations, Freehold, NJ, USA), according to the instructions of the manufacturer.

The purified sequences were denatured using HiDi deionized formamide (Applied Biosystems): the denaturation protocol consisted of 5 min at 95°C. Sequences were then analyzed by capillary electrophoresis, using the AB 3500 Genetic Analyzer (Applied Biosystems). The obtained sequences were evaluated using the Variant Reporter Software v1.1 (Applied Biosystems).

Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) or as mean ± standard deviation (SD), depending on their distribution, while categorical variables were described as frequencies. Continuous variables were compared by the analysis of variance (ANOVA) or the Kruskal-Wallis test depending on their distribution among the groups.

The heterozygosity (HET) was determined and the Hardy-Weinberg equilibrium (HWE) test was performed through the chi-square (χ^2) goodness-of-fit test, for each SNP in each group.

For each SNP, the allele frequencies and genotype frequencies were calculated.

Genetic associations were calculated by the "per-genotype analysis", "per-allele analysis" and "linear trend analysis" tests, using the SAS software version 9.1.4 (SAS Institute Inc., Cary, NC, USA).

Statistical significance was evaluated by the two-tailed t-test ($p < 0.05$).

Sample size analysis

The sample size was calculated using the QUANTO program version 1.2.3 [$\alpha = 5\%$ and power $(1\text{-}\beta) = 80\%$]; the calculated sample size stated a number of at least 69 for each group.

Results

Characteristics of the population

In this study, we reached the minimum number of cases required according to the sample size test. We enrolled 253 Caucasian individuals: 74 in the case group (patients with at least one AR event after KT), 109 in the control I group

Table 1: Characteristics of the primers for each SNP.

Gene	SNP	Forward primer (5′ → 3′)	Reverse primer (5′ →3′)	Length, bp
IL-10	rs1800872	GCGTGTTCCTAGGTCACAGT	ACTCTTACCCACTTCCCCCA	369
TNF	rs1800629	GCCAAGACTGAAACCAGCAT	TTGGGGACACACAAGCATCA	515
	rs1045642	AGTGTGGCCAGATGCTTGTA	CTGCCTACCACATGCATACAT	593
ABCB1	rs1128503	CAACATCAGAAAGATGTGCAA	TGAGTTGGCCATCTATCCACC	615
	rs2032582	GTCCAAGAACTGGCTTTGCT	GCATGAGTTGTGAAGATAATA	446
UGT1A9	rs2741045	CACAGGCGAGCCCCAATTTA	GGTGGGAGAAATACCAGCACA	452
	rs2741046			
	rs6714486			
UGT1A9	rs17868320	ACAGAGTCGTGCTGTTTTGC	AGGTCAAGGTGGGCGTATC	237
IMPDH2	rs11706052	ACAGGAAAGTTGCCCATTGT	TCCTAGGACAAGAAGTAAGTCTCAG	283
IMPDH1	rs2278293	TTACACACCTGCATGGGGAC	TCCGTGATGAAGCCCTGTTC	496
	rs2278294			

ABCB1, adenosine triphosphate-binding cassette sub-family B member 1; bp, base pair; *IL-10*, interleukin-10; *IMPDH1*, inosine monophosphate dehydrogenase 1; *IMPDH2*, inosine monophosphate dehydrogenase 2; SNP, single-nucleotide polymorphism; *TNF*, tumor necrosis factor; *UGT1A9*, uridine diphosphate glucuronosyltransferase family 1 member A9.

(patients without any AR episode after KT) and 70 in the control II group (healthy blood donors). Comparing the gender between the three groups, we found a statistically significant difference: case 74.32% M vs. control I 61.47% M vs. control II 77.14% ($p = 0.048$).

Also, comparing the median age, we found a statistically significant difference between the three groups: case: median, 53 (IQR, 43–62) years vs. control I: 54 (47–62) years vs. control II: 49 (41–54) years ($p = 0.019$). However, the difference comparing the case and control II groups and the case and control I groups was not statistically significant ($p = 0.09$ and $= 0.83$, respectively); only between the control I and control II groups there was a significant difference ($p = 0.01$). Interpreting these data, the transplanted population (case and control I) may be considered homogeneous for age.

Genotyping

Each SNP respects the HWE (p-value > 0.05) in the three groups and so the population did not have any factor that may alter this equilibrium.

We calculated the HET for each SNP separately for each group, as shown in Table 2. The p-value for the HWE was also reported.

Allele frequencies

We calculated the allele frequencies for each SNP in the three groups. The allele frequencies of the three groups were superimposable, as shown in Table 3.

The allele frequencies of the case group were in general superimposable with those of the worldwide, European and Italian populations [14].

All the allele frequencies related to the control II group were very similar to those of the worldwide population, confirming this control group was really representative of the general population.

We did not find any statistically significant difference between the allele frequencies of the case and control I groups ($p > 0.05$) by "per-allele analysis" and grouping transplanted patients (case and control I) comparing with the control II group (which ideally represents the general population).

Genotype frequencies

The genotype frequencies determined in the three groups were similar: the most frequent polymorphism in a group was the same in the other groups (Table 4).

There was no statistically significant difference comparing the genotype frequencies of the case and control I groups (Table 5) and comparing the case and control II groups ($p > 0.05$) by "per-genotype analysis" and by "linear trend analysis".

No statistically significant difference was found even grouping transplanted patients (case and control I groups) vs. control II group ($p > 0.05$).

Moreover, in the control I group, no subject was homozygous G/G for the SNP rs11706052 of the *IMPDH2* gene. Likewise, in the control II group, no one was heterozygous A/G and A/T for the SNP rs2032582 of the *ABCB1*

Table 2: HET evaluation and HWE in the case, control I and control II groups.

Gene	SNP	Chromosome	Case		Control I		Control II	
			Observed HET	HWE p-value	Observed HET	HWE p-value	Observed HET	HWE p-value
IL-10	rs1800872	Chr1	0.34	0.83	0.39	0.72	0.44	0.90
TNF	rs1800629	Chr6	0.19	0.87	0.22	0.62	0.26	0.68
ABCB1	rs1128503	Chr7	0.53	0.15	0.50	0.45	0.43	0.33
ABCB1	rs2032582	Chr7	0.53	0.82	0.48	0.10	0.39	0.13
ABCB1	rs1045642	Chr7	0.53	0.63	0.52	0.51	0.49	0.81
UGT1A9	rs2741045	Chr2	0.38	0.78	0.36	0.18	0.41	0.82
UGT1A9	rs2741046	Chr2	0.38	0.78	0.36	0.18	0.40	0.92
UGT1A9	rs6714486	Chr2	0.10	0.67	0.05	0.81	0.09	0.71
UGT1A9	rs17868320	Chr2	0.09	0.71	0.04	0.85	0.07	0.76
IMPDH2	rs11706052	Chr3	0.20	0.91	0.22	0.19	0.14	0.52
IMPDH1	rs2278293	Chr7	0.46	0.63	0.53	0.48	0.53	0.63
IMPDH1	rs2278294	Chr7	0.44	0.71	0.52	0.25	0.39	0.20

ABCB1, adenosine triphosphate-binding cassette sub-family B member 1; HET, heterozygosity; HWE, Hardy-Weinberg equilibrium; *IL-10*, interleukin-10; *IMPDH1*, inosine monophosphate dehydrogenase 1; *IMPDH2*, inosine monophosphate dehydrogenase 2; SNP, single-nucleotide polymorphism; *TNF*, tumor necrosis factor; *UGT1A9*, uridine diphosphate glucuronosyltransferase family 1 member A9.

Table 3: Allele frequencies of the worldwide, European and Italian populations for the case group, control I group and control II group.

Gene	SNP	Chromosome	Allele	Worldwide pop.	European pop.	Italian pop.	Case	Control I	Control II
IL-10	rs1800872	Chr1	G	0.57	0.76	0.71	0.78	0.73	0.68
			T	0.43	0.24	0.29	0.22	0.28	0.32
TNF	rs1800629	Chr6	G	0.91	0.87	0.91	0.89	0.88	0.86
			A	0.09	0.13	0.09	0.11	0.12	0.14
ABCB1	rs1128503	Chr7	C	0.58	0.58	0.58	0.66	0.63	0.59
			T	0.42	0.42	0.42	0.35	0.37	0.41
ABCB1	rs2032582	Chr7	G	0.62	0.57	0.58	0.64	0.64	0.60
			T	0.33	0.41	0.40	0.35	0.35	0.40
			A	0.05	0.02	0.02	0.02	0.01	–
ABCB1	rs1045642	Chr7	C	0.60	0.48	0.53	0.59	0.56	0.5
			T	0.40	0.52	0.47	0.41	0.44	0.5
UGT1A9	rs2741045	Chr2	C	0.84	0.73	0.72	0.75	0.71	0.69
			T	0.16	0.27	0.28	0.25	0.29	0.31
UGT1A9	rs2741046	Chr2	T	0.84	0.73	0.72	0.75	0.71	0.73
			C	0.16	0.27	0.28	0.25	0.29	0.27
UGT1A9	rs6714486	Chr2	T	0.93	0.95	0.95	0.95	0.98	0.96
			A	0.07	0.05	0.05	0.05	0.02	0.04
UGT1A9	rs17868320	Chr2	C	0.98	0.95	0.95	0.96	0.98	0.96
			T	0.02	0.05	0.05	0.04	0.02	0.04
IMPDH2	rs11706052	Chr3	A	0.95	0.88	0.88	0.89	0.89	0.93
			G	0.05	0.12	0.12	0.11	0.11	0.07
IMPDH1	rs2278293	Chr7	G	0.57	0.53	0.56	0.59	0.52	0.51
			A	0.43	0.47	0.44	0.41	0.48	0.49
IMPDH1	rs2278294	Chr7	G	0.56	0.65	0.66	0.64	0.62	0.65
			A	0.44	0.35	0.34	0.36	0.38	0.35

ABCB1, adenosine triphosphate-binding cassette sub-family B member 1; *IL-10*, interleukin-10; *IMPDH1*, inosine monophosphate dehydrogenase 1; *IMPDH2*, inosine monophosphate dehydrogenase 2; SNP, single-nucleotide polymorphism; *TNF*, tumor necrosis factor; *UGT1A9*, uridine diphosphate glucuronosyltransferase family 1 member A9.

gene and homozygous G/G for the SNP rs11706052 of the *IMPDH2* gene.

Finally, there was no statistically significant genetic association comparing the allele and genotype frequencies of the three groups by "per-allele analysis", "per-genotype analysis" and "linear trend analysis".

Discussion

In this study, we analyzed several SNPs of the *IL-10*, *TNF*, *ABCB1*, *UGT1A9*, *IMPDH1* and *IMPDH2* genes to determine their association with AR after KT; we tried to identify an allele variant specifically associated with AR that could be an interesting marker supporting the evaluation of immunosuppressive therapy.

In our previous study [15], the results showed that patients with the C allele in the SNP rs1045642 and the A allele in the SNP rs2032582 of the *ABCB1* gene had more frequent AR episodes, but the sample size was reached only for the two control groups, and not for the case group. In the actual analysis, we had the correct sample size for each group.

The literature data state that male individuals are in general more affected by AR after KT than females [16]. Our data about these confirm gender-related differences, in all the three analyzed groups.

Also, for the age of the individuals between the three groups, we saw a statistically significant difference ($p = 0.019$). However, there was no statistically significant difference between the case and control II groups ($p = 0.09$) and between the case and control I groups ($p = 0.83$); there was a significant difference only between the control I and control II groups ($p = 0.01$). These data suggest that the transplanted population (case and control I groups) may be considered homogeneous for age.

Each SNP respects the HWE, in each group. Conversely, in the preliminary analysis [15], the SNP rs1045642 of the *ABCB1* gene did not respect the HWE in the group of individuals with AR, and so it seems possible a connection between the C allele variant (the one with the highest frequency) and the major susceptibility to the AR event. On the contrary, the correct sample size did not

Table 4: Genotype frequencies.

Gene	SNP	Chromosome	Genotype	Case (frequency)	Control I (frequency)	Control II (frequency)
IL-10	rs1800872	Chr1	G/G	0.61	0.53	0.46
			G/T	0.34	0.39	0.44
			T/T	0.05	0.08	0.10
TNF	rs1800629	Chr6	G/G	0.80	0.77	0.73
			A/G	0.19	0.22	0.26
			A/A	0.01	0.01	0.01
ABCB1	rs1128503	Chr7	C/T	0.53	0.50	0.43
			C/C	0.39	0.38	0.37
			T/T	0.08	0.12	0.20
ABCB1	rs2032582	Chr7	G/T	0.49	0.45	0.39
			G/G	0.38	0.40	0.41
			T/T	0.09	0.12	0.20
			A/G	0.03	0.02	–
			A/T	0.01	0.01	–
ABCB1	rs1045642	Chr7	C/T	0.53	0.52	0.48
			C/C	0.32	0.30	0.26
			T/T	0.15	0.18	0.26
UGT1A9	rs2741045	Chr2	C/C	0.56	0.53	0.49
			C/T	0.38	0.36	0.41
			T/T	0.06	0.11	0.10
UGT1A9	rs2741046	Chr2	T/T	0.56	0.53	0.53
			C/T	0.38	0.36	0.40
			C/C	0.06	0.11	0.07
UGT1A9	rs6714486	Chr2	T/T	0.90	0.95	0.91
			A/T	0.10	0.05	0.09
UGT1A9	rs17868320	Chr2	C/C	0.92	0.96	0.93
			C/T	0.08	0.04	0.07
IMPDH2	rs11706052	Chr3	A/A	0.79	0.78	0.86
			G/A	0.20	0.22	0.14
			G/G	0.01	–	–
IMPDH1	rs2278293	Chr7	A/G	0.46	0.54	0.53
			G/G	0.36	0.25	0.24
			A/A	0.18	0.21	0.23
IMPDH1	rs2278294	Chr7	A/G	0.44	0.52	0.39
			G/G	0.42	0.36	0.46
			A/A	0.14	0.12	0.15

ABCB1, adenosine triphosphate-binding cassette sub-family B member 1; *IL-10*, interleukin-10; *IMPDH1*, inosine monophosphate dehydrogenase 1; *IMPDH2*, inosine monophosphate dehydrogenase 2; SNP, single-nucleotide polymorphism; *TNF*, tumor necrosis factor; *UGT1A9*, uridine diphosphate glucuronosyltransferase family 1 member A9.

confirm this data and did not show any other significant connection.

We examined the allele frequencies for each SNP, and the emerging data showed a homogeneous distribution in the three groups. We compared the same frequencies with those of the Italian, European and worldwide population, and in general they were all superimposable [14].

We calculated the genotype frequencies for each SNP in the groups: these data did not show any statistically significant difference. Interestingly, there was not any A/G and A/T heterozygous individual for the SNP rs2032582 of the *ABCB1* gene, and in the two control groups there was not any G/G homozygous individual for the SNP rs117066052 of the *IMPDH2* gene.

Finally, we calculated genetic associations by comparing the allele frequencies and genotype frequencies of each group; the comparisons were as follows: case vs. control I, case vs. control II, case plus control I (transplanted group) vs. control II. We found no statistically significant difference from any of these comparisons ($p > 0.05$).

The studied polymorphisms were selected because some evidences of the literature show a possible connection with the AR event after KT [10, 13].

Table 5: Genetic associations of the case group vs. the control I group.

Gene	SNP	Chromosome	p-Value Per-genotype analysis	p-Value Per-allele analysis	p-Value Linear-trend analysis
IL-10	rs1800872	Chr1	0.54	0.26	0.27
TNF	rs1800629	Chr6	0.85	0.74	0.74
ABCB1	rs1128503	Chr7	0.70	0.62	0.59
ABCB1	rs2032582	Chr7	0.95	0.89	0.89
ABCB1	rs1045642	Chr7	0.89	0.65	0.64
UGT1A9	rs2741045	Chr2	0.43	0.37	0.39
UGT1A9	rs2741046	Chr2	0.43	0.37	0.39
UGT1A9	rs6714486	Chr2	0.18	0.19	0.18
UGT1A9	rs17868320	Chr2	0.17	0.18	0.17
IMPDH2	rs11706052	Chr3	0.44	0.96	0.96
IMPDH1	rs2278293	Chr7	0.33	0.22	0.22
IMPDH1	rs2278294	Chr7	0.57	0.79	0.78

ABCB1, adenosine triphosphate-binding cassette sub-family B member 1; *IL-10*, interleukin-10; *IMPDH1*, inosine monophosphate dehydrogenase 1; *IMPDH2*, inosine monophosphate dehydrogenase 2; SNP, single-nucleotide polymorphism; *TNF*, tumor necrosis factor; *UGT1A9*, uridine diphosphate glucuronosyltransferase family 1 member A9.

IL-10 and *TNF* encode for two cytokines involved in the inflammation process, and some scientific evidences describe a reduction in the *IL-10* blood levels after transplantation because of the immunosuppressive therapies [17, 18]. However, the majority of the studies published in the literature did not show any statistically significant difference by connecting the SNPs located in these two genes with the risk of AR, and our results confirmed this trend [19–24].

UGT1A9 encodes for an enzyme involved in glucuronidation, which converts small lipid molecules into hydrophilic metabolites that are simple to remove, and in its activity, it also metabolizes MPA used for immunosuppression. Some studies suggest that some polymorphisms located in this gene promote the glucuronidation of MPA, with a subsequent reduction in drug levels in the individuals after transplantation [25–28]. In our study, comparing these SNPs between the case and control I groups, we did not confirm these findings.

IMPDH2 encodes for an enzyme responsible of the de novo synthesis of guanine nucleotides and it is the target of MPA. In the previous analysis, many different polymorphisms of this gene were investigated, but in almost all there was no HET: in each population, there were just one allele variant and one genotype variant [15]. The only SNP that showed different allele and genotype variants was rs11706052: certain studies found a correlation between the G variant and a lower response to MPA, by comparison with the A/A genotype [28, 29]. Because of this, the actual analysis was conducted only for the SNP rs11706052. Also, for this SNP, the data revealed no variant specifically connected with AR.

IMPDH1 encodes for an enzyme which catalyzes the key step in the de novo synthesis of guanine nucleotides, and this enzyme is the main target of MPA, widely used to prevent rejection in solid organ transplantations. This gene, initially not studied, was enrolled in the analysis because a study found two SNPs (rs2278293 and rs2278294) highly associated with biopsy-proven AR within the first year after transplantation, because it seemed that it might contribute to determine the differences in the response and in the toxicity to MPA in transplanted patients [30]. The results of our statistical analysis, instead, did not show any significant association between the analyzed SNPs and the AR after KT.

The *ABCB1* gene encodes for a transmembrane efflux pump, which may expel various xenobiotics, and so immunosuppressive drugs are used for the prevention of AR after KT. Many studies on various therapeutic agents have shown how people with the C/C and C/T genotypes are considered “high pumpers” (with a major activity of the pump), while people with the T/T genotype are “low pumpers” [31]. In effect, the lower *ABCB1* activity related to the T variant is associated with a significant increase in the concentration of intracellular cyclosporine, and this exposes patients to a higher risk of toxicity caused by drug accumulation [32]. Other studies have shown that an increase in the P-glycoprotein activity may cause a reduced intracellular exposure of T lymphocytes to the cyclosporine, which cannot explain its inhibitory action and because of this, it is connected with the AR event [33]. This hypothesis can also be found in some lung transplant studies, where the C/C and C/T genotypes appear more connected with persistent AR than the T/T variant [34], and also in neurodegenerative diseases, where the

C variant seems to be connected with a higher risk of the development of Alzheimer's disease [35]. The results of our previous study [15] seemed to confirm the C allele of risk, because this was the major represented allele in the case group, and in addition, the SNP rs1045642 did not respect the HWE. Our actual analysis, with the complete sample size, did not confirm this hypothesis, because there was no statistically significant difference between the groups, and the SNP rs1045642 respects the HWE in the three populations that we studied. Nevertheless, the C allele remained predominant.

It is clear that with this study we highlight a fundamental aspect of scientific research generally presumed, that is the sample size of groups/populations enrolled for a scientific study. Our actual experience shows that if we do not meet the required sample size, we cannot declare any result with any certainty, because that result may be very different from the data otherwise obtained with the achievement of sample size, and so it could not have any value representative of the reality.

Eventually, the actual analysis shows the importance of the analysis and the achievement of sample size to evaluate the associations between polymorphic variants and the studied event. In the previous study [15], the case group patients and the control II individuals were compared, with the assumption that transplanted patients of the control I group may reject the organ. From this comparison, it was seen that the allele and genotype associations of the two variants (rs1045642 and rs2032582) of the *ABCB1* gene were statistically significant (rs1045642: per-genotype $p = 0.01$, per-allele $p = 0.01$, linear trend $p = 0.01$; rs2032582: per-genotype $p = 0.02$, per-allele $p = 0.03$, linear trend $p = 0.04$). In contrast, with the achievement of sample size, the trend of the previous data was not confirmed.

For a future personalized therapy, the analysis of polymorphic variants related to drug metabolism in relation to drug doses could be interesting. The result could be a useful instrument for clinicians to set up anti-rejection therapy.

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