

# IMPDH1 Gene Polymorphisms and Association With Acute Rejection in Renal Transplant Patients

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Inosine 5'-monophosphate dehydrogenase 1 (IMPDH1) catalyzes the rate-limiting step of the *de novo* pathway for purine synthesis and is a major target of the immunosuppressive drug mycophenolic acid (MPA). Few variants of the *IMPDH1* gene have been reported. The objective of this study was to identify and characterize *IMPDH1* variants to determine whether genetic variation contributes to differences in MPA response and toxicity in transplant patients. Seventeen genetic variants were identified in the *IMPDH1* gene with allele frequencies ranging from 0.2 to 42.7%. In this study, 191 kidney transplant patients who received mycophenolate mofetil were genotyped for *IMPDH1*. Two single-nucleotide polymorphisms, rs2278293 and rs2278294, were significantly associated with the incidence of biopsy-proven acute rejection in the first year post-transplantation. Future studies of the multifactorial nature of acute rejection must consider *IMPDH1* polymorphisms in MPA-treated patients.

Inosine 5'-monophosphate dehydrogenase (IMPDH) (EC 1.1.1.205) is the rate-limiting enzyme in the pathway of *de novo* biosynthesis of guanine by driving inosine monophosphate (IMP) to xanthosine monophosphate with the reduction of nicotinamide adenine dinucleotide (NAD). This enzyme is the target of mycophenolic acid (MPA), the active constituent of the prodrug mycophenolate mofetil (MMF), which is widely used clinically to prevent allograft rejection following solid organ transplantation.<sup>1</sup> MPA is also used as an immunomodulator in immune-related diseases such as AIDS, lupus nephritis, myasthenia gravis, and immune thrombocytopenic purpura.<sup>2-6</sup> Unlike other cell types, which can use the salvage pathway for purine synthesis, lymphocytes are exclusively dependent upon the *de novo* pathway catalyzed by IMPDH for the generation of guanosine nucleotide.<sup>7,8</sup> MPA uncompetitively, selectively, and reversibly inhibits this pathway and therefore inhibits cell proliferation.

IMPDH has two isoforms named IMPDH1 and IMPDH2, and the genes that encode these proteins are located on two different chromosomes.<sup>9</sup> *IMPDH1* is located on chromosome 7 region q32.1 and is constitutively expressed in all tissues.

IMPDH1 shares 84% amino-acid homology with IMPDH2<sup>10,11</sup> and has substrate affinities and  $K_i$  values that are virtually indistinguishable.<sup>12</sup> In an *IMPDH1*-knockout mouse model, *IMPDH1* deletion inhibited T-cell activation in response to anti-CD3 and anti-CD28 antibodies.<sup>13</sup> Although MPA has been shown to inhibit the activities of both IMPDH1 and IMPDH2 isoforms *in vitro*, IMPDH2 is 4.8 times more sensitive to MPA inhibition than IMPDH1.<sup>14</sup>

Several mutations in the IMPDH-binding site confer variable degrees of resistance to MPA in the parasite *Trichomonas foetus*, and varying drug-sensitive forms of this enzyme have been detected in *Saccharomyces cerevisiae*. However, few studies have been reported on *IMPDH* gene variants in humans.<sup>15-17</sup> Recent findings have shown that mutations in *IMPDH1* cause autosomal-dominant retinitis pigmentosa.<sup>18,19</sup> No other changes in *IMPDH1* that result in a change in the genotype-phenotype relationship have been described.

As maintenance of the balance between rejection and over-immunosuppression is difficult, pharmacokinetic and pharmacodynamic monitoring of MMF have been proposed to optimize drug dosage and the administration schedule.<sup>20</sup>

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Hale *et al.*<sup>21</sup> previously reported that the area under curve (AUC) of MPA is inversely associated with the likelihood of allograft rejection after renal transplantation in patients receiving MMF. The relationship between MMF pharmacokinetic parameters and the risk of adverse effects is not as well defined. Considering the significant variability in the effects of a given concentration of MPA, the therapeutic range for the desired pharmacologic effect without adverse effects in most patients is difficult to discern.<sup>22</sup> Interindividual variability in IMPDH activity has been observed in healthy volunteers as well as transplant patients.<sup>23,24</sup> Pharmacodynamic monitoring of IMPDH activity has been proposed, and a significant relationship has been reported between pretransplant IMPDH activity and clinical outcome after renal transplantation.<sup>24</sup> However, MPA is most frequently administered to transplant patients without monitoring MPA plasma concentrations, IMPDH concentrations, or other relevant gene polymorphisms.

The considerable variability in baseline IMPDH activity and MPA response may logically be under the control of genetic variation within the *IMPDH* genes or in gene expression. Analysis of genetic variants could provide the explanation for the variability of IMPDH activity and MMF response in transplant patients. Therefore, the objective of this study was to identify *IMPDH1* genetic variants in renal transplant patients and to retrospectively look for the association of these polymorphisms with leukopenia or biopsy-proven acute rejection in renal transplant patients receiving MPA.

## RESULTS

To examine *IMPDH1* genetic variation, 17 exons and the intron–exon boundary regions were initially sequenced from 30 genomic DNA samples from liver transplant patients. Seventeen genetic variants were identified in the *IMPDH1* gene. Eleven single-nucleotide polymorphisms (SNPs), including four exonic variants and seven intronic SNPs with relatively high allele frequencies, were subjected to TaqMan SNP assay using an ABI 7900 Real-Time PCR System. The identified polymorphisms and their frequencies are listed in **Table 1**. **Table 2** lists the demographic data for the 191 renal transplant patients who were divided into patients with and without an episode of biopsy-proven acute rejection in the first postoperative year. No significant demographic difference was found between the group with rejection and without rejection with respect to age at transplantation, weight, height, gender, and race/ethnicity ( $P > 0.05$ ). All of the SNPs genotyped were in the Hardy–Weinberg equilibrium ( $P > 0.05$  observed vs predicted).

Two novel non-synonymous variants, Asp301Asn and Gly519Arg, were identified in exons 10 and 15 of *IMPDH1*. The effects of these polymorphisms were determined on IMPDH production (see **Supplementary Material, Table S4** and **Figures S3** and **S4**), and neither was found to change IMPDH activity or binding.

Biopsy-proven acute rejection occurred in 15% of patients in the first postoperative year, which approximates the rate of rejection observed currently in transplant centers in the United States with a high percentage of high-risk patients.<sup>25</sup>

**Table 1 Genetic variants of *IMPDH1* in transplant patients detected by bidirectional DNA sequencing**

| Variants <sup>a</sup> | Reported ID <sup>b</sup> | Position  | Flanking sequence          | AA change <sup>c</sup> | Minor allele frequency (%) | Reported <sup>b</sup> |
|-----------------------|--------------------------|-----------|----------------------------|------------------------|----------------------------|-----------------------|
| IVS1+109A>T           | rs2288553 <sup>d</sup>   | Intron 1  | GGCCCCACAA[A/T]CCCTTCCCCT  |                        | 22.51                      | 35.2                  |
| IVS3+1149A>G          | rs11770116               | Intron 3  | GTCTTCAGGG[A/G]ACAACAGCCC  |                        | 20.68                      | 37.5                  |
| IVS5–354A>G           | rs2288548 <sup>d</sup>   | Intron 4  | ATCAAGATTG[A/G]GAAGTTTCA   |                        | 15.97                      | 22.9                  |
| IVS5–227C>T           | rs2288549 <sup>d</sup>   | Intron 4  | GGTACCCAG[C/T]AGACCTTTCG   |                        | 17.07                      | 5.7                   |
| IVS6–169T>C           | rs4731448                | Intron 5  | TGTCTGTCAT[T/C]TTTCTTTCCA  |                        | 17.54                      |                       |
| IVS7–34C>A            |                          | Intron 6  | GAGGAATGGG[C/A]CCCGGCTCTG  |                        | 1.8 <sup>e</sup>           |                       |
| IVS7+125G>A           | rs2278293 <sup>d</sup>   | Intron 7  | TCCTCTCAGT[G/A]GAGCCTTGGG  |                        | 42.67                      | 37.6                  |
| IVS8–106G>A           | rs2278294 <sup>d</sup>   | Intron 7  | CCCACTGAGG[G/A]TCCTGGCTTC  |                        | 40.58                      | 32.2                  |
| 898G>A                |                          | Exon 10   | TCAATGATTGC[G/A]ATGAGCTGGT | <b>Asp301Asn</b>       | 0.29                       |                       |
| 984C>G                | rs2288550                | Exon 10   | GAAGCAGCT[C/G]CTCTGTGGGG   | Leu329Leu              | 9.41                       | 20.8                  |
| IVS11–22G>A           |                          | Intron 10 | TAAGCCCAA[G/A]CTCTTCCGTC   |                        | 0.5 <sup>e</sup>           |                       |
| IVS13+33C>T           |                          | Intron 13 | TTGGGAGG[C/T]GAGGGCTGGA    |                        | 13.5 <sup>e</sup>          |                       |
| IVS15–114T>G          |                          | Intron 14 | ACTCAGCCCAG[T/G]GAGGGGTGGC |                        | 7.3 <sup>e</sup>           |                       |
| 1552G>A               |                          | Exon 15   | CGCAGCGAGG[G/A]GGATAAAGTG  | <b>Gly519Arg</b>       | 0.88                       |                       |
| 1572C>T               | rs2228075 <sup>d</sup>   | Exon 15   | TGAAGATCGC[C/T]CAGGGTGTCT  | Ala525Ala              | 25.39                      |                       |
| IVS15+33C>T           |                          | Intron 15 | GCTCCTGCTT[C/T]CTTCTCTCG   |                        | 3.9 <sup>e</sup>           |                       |
| IVS15+43G>A           |                          | Intron 15 | CCTTCTCTC[G/A]CTCTTCTCCA   |                        | 6.7 <sup>e</sup>           |                       |

IMPDH, inosine 5'-monophosphate dehydrogenase; SNP, single-nucleotide polymorphism. <sup>a</sup>The position is relative to ATG start site with the A as nucleotide +1 and based on mRNA sequence from GenBank accession number NM\_000883. <sup>b</sup>The frequencies were reported by GenBank. <sup>c</sup>Amino-acid (AA) change with its position shown in middle (NP\_000874). Non-synonymous change is shown in bold. <sup>d</sup>Tagging SNPs. <sup>e</sup>Frequencies based on sequencing in transplant patients.

**Table 2 Demographic characteristics of the kidney transplant patients**

|                            | No rejection | Rejection   | P-values | No leukopenia | Leukopenia  | P-values |
|----------------------------|--------------|-------------|----------|---------------|-------------|----------|
| N                          | 161          | 29          |          | 129           | 60          |          |
| Height (inch) <sup>a</sup> | 65.7 ± 7.0   | 64.8 ± 4.7  | 0.51     | 65.8 ± 7.6    | 65.0 ± 4.1  | 0.44     |
| Weight (kg) <sup>a</sup>   | 68.7 ± 16.6  | 63.1 ± 15.5 | 0.09     | 68.2 ± 16.6   | 67.0 ± 16.4 | 0.65     |
| Age (years) <sup>a</sup>   | 46.5 ± 12.6  | 43.7 ± 14.6 | 0.29     | 45.7 ± 13.1   | 46.7 ± 12.5 | 0.62     |
| MPA (mg/kg)                | 5.0 ± 2.3    | 5.8 ± 2.8   | 0.09     | 5.2 ± 2.5     | 4.8 ± 2.2   | 0.37     |
| <b>Gender</b>              |              |             |          |               |             |          |
| Female                     | 69           | 13          | 1.00     | 56            | 26          | 1.00     |
| Male                       | 91           | 16          |          | 73            | 34          |          |
| <b>Race</b>                |              |             |          |               |             |          |
| Asian                      | 29           | 5           |          | 22            | 12          |          |
| Caucasian                  | 23           | 2           |          | 15            | 10          |          |
| Hispanic                   | 81           | 15          | 0.80     | 70            | 26          | 0.65     |
| African American           | 16           | 4           |          | 13            | 7           |          |
| Others                     | 12           | 3           |          | 9             | 6           |          |

MPA, mycophenolic acid. <sup>a</sup>Data were represented as mean ± SD.

Two SNPs, rs2278293 and rs2278294, were significantly associated with the incidence of biopsy-proven acute rejection in the first year post-transplantation (Table 3,  $P < 0.05$ ,  $\chi^2$ ). The prognostic value of each of these SNPs in predicting biopsy-proven rejection exceeded 50% for both the positive predictive value and the negative predictive value (Table 4). Both of these SNPs are intronic, and one is a component of a haplotype block. The odds ratio by logistic regression analysis was 0.34 for SNP rs2278293 (95% confidence interval: 0.15–0.76;  $P = 0.008$ ) and 0.40 for rs2278294 (95% confidence interval: 0.18–0.89;  $P = 0.02$ ).

Leukopenia occurred in approximately 32% of the patients receiving MPA in the first postoperative year (Table 3). In previous reports of leukopenia in MPA-treated transplant patients, leukopenia occurs in 25.9–39.3% of patients, depending on the criteria for classifying leukopenia.<sup>26</sup> Leukopenia was not associated with any of the *IMPDH1* SNPs. We observed leukopenia in the one variant *IMPDH2* L263F patient in accordance with our previous observations.<sup>27</sup>

Six tagging SNPs were selected to present the two haplotype blocks in *IMPDH1* (Table 1 and Figure 1). Two haplotypes, rs11770116–rs2288548 and rs2278294–rs2228075, were also examined for their influence on leukopenia or acute rejection within 1-year post-transplantation (Figure 1). No haplotype association was found for leukopenia or acute rejection.

The genotype distribution for all of the SNPs, with one exception, was not statistically different between the Hispanic and non-Hispanic patients (Table S2). The distribution was significantly different for rs2288553, but this SNP was not significantly associated with either acute rejection or leukopenia (Table S3).

## DISCUSSION

Numerous SNPs have now been associated with acute rejection after renal transplantation. Most of these SNPs have been part of the transplant immune response and include cytokines, chemokines, adhesion molecules, and growth factors. The pharmacogenetic factors affecting drug bioavailability, direct action, and disposition in organ transplant patients have not been associated with acute rejection previously, with the exception of an *ABCB1* polymorphism.<sup>28</sup>

Plasma concentrations of MPA are affected by a number of gene polymorphisms. Both the glucuronidating uridine 5'-diphospho-glucuronosyltransferase (UGT) enzymes<sup>29–31</sup> and the drug transporter multidrug resistance-associated protein-2 (MRP2)<sup>32</sup> polymorphisms may affect MPA plasma concentrations. P-glycoprotein, MRP2, and the organic anion-transporting polypeptide (OATP) transporters may play a potential role in MPA disposition, so it is not surprising that considerable variability in MPA pharmacokinetics has been observed in transplant patients.<sup>33</sup> Figure 2 shows that the critical step in exerting the effect of MPA is at *IMPDH*, which is controlled by *IMPDH1* and *IMPDH2*.

Our previous explorative study showed that *IMPDH2* is more conserved than its isoform *IMPDH1*. Although we identified a novel variant L263F that affects enzyme activity in *IMPDH2*,<sup>27</sup> its rare frequency precludes it from having a large effect in a small patient population. Another SNP, rs11706052, was not associated with the incidence of biopsy-proven rejection in this study. Owing to the low frequency of the L263F variant, further studies in larger populations would be needed to define its impact on clinical outcome.

The *IMPDH* monomer contains eight-stranded  $\alpha/\beta$  barrels, which are the core domain of the *IMPDH1* protein.

**Table 3** The association of *IMPDH1* and *IMPDH2* SNPs with biopsy-proven rejection and leukopenia in kidney transplant patients

| ID            | Genotype | Total | No rejection | Rejection | No leukopenia | Leukopenia |
|---------------|----------|-------|--------------|-----------|---------------|------------|
| <i>IMPDH1</i> |          |       |              |           |               |            |
| rs2288553     | AA       | 118   | 99           | 19        | 79            | 39         |
|               | AT+TT    | 72    | 62           | 10        | 50            | 22         |
| r11770116     | AA       | 124   | 106          | 18        | 87            | 37         |
|               | AG+GG    | 66    | 55           | 11        | 42            | 24         |
| rs2288548     | AA       | 134   | 115          | 19        | 94            | 40         |
|               | AG+GG    | 56    | 46           | 10        | 35            | 21         |
| rs2288549     | CC       | 138   | 120          | 18        | 95            | 43         |
|               | CT+TT    | 52    | 41           | 11        | 34            | 18         |
| rs4731448     | TT       | 135   | 116          | 19        | 94            | 41         |
|               | TC+CC    | 55    | 45           | 10        | 35            | 20         |
| rs2278293*    | GG       | 69    | 52           | 17        | 43            | 26         |
|               | GA+AA    | 121   | 109          | 12        | 86            | 35         |
| rs2278294*    | GG       | 69    | 53           | 16        | 44            | 25         |
|               | GA+AA    | 121   | 108          | 13        | 85            | 36         |
| rs2228075     | CC       | 105   | 85           | 20        | 72            | 33         |
|               | CT+TT    | 85    | 76           | 9         | 57            | 28         |
| 898G > A      | GG       | 168   | 144          | 24        | 115           | 53         |
|               | GA       | 1     | 1            | 0         | 0             | 1          |
| rs2288550     | CC       | 137   | 117          | 20        | 94            | 43         |
|               | CG       | 32    | 28           | 4         | 21            | 11         |
| 1552G > A     | GG       | 166   | 143          | 23        | 114           | 52         |
|               | GA       | 3     | 2            | 1         | 1             | 2          |
| <i>IMPDH2</i> |          |       |              |           |               |            |
| 787C > T      | CC       | 168   | 144          | 24        | 115           | 53         |
|               | CT       | 1     | 1            | 0         | 0             | 1          |
| rs11706052    | TT       | 153   | 132          | 21        | 101           | 52         |
|               | TC+CC    | 16    | 13           | 3         | 14            | 2          |

IMPDH, inosine 5'-monophosphate dehydrogenase; SNP, single-nucleotide polymorphism. \* $P < 0.05$  for rejection by  $\chi^2$ .

**Table 4** Prognostic value<sup>a</sup> of the *IMPDH1* polymorphism for determining biopsy-proven rejection in kidney transplant patients treated with MPA

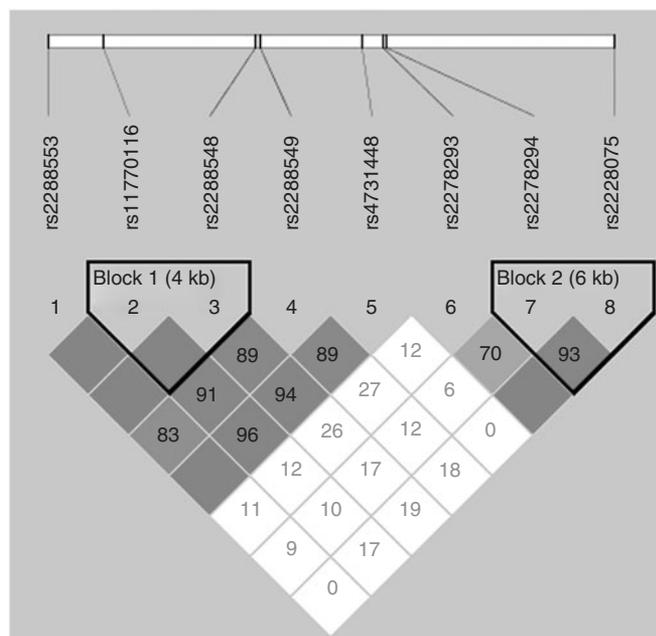
|           | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) |
|-----------|-----------------|-----------------|-------------------------------|-------------------------------|
| rs2278293 | 24.63           | 90.08           | 58.62                         | 67.7                          |
| 95% CI    | (15–36)         | (83–94)         | (38–76)                       | (59–74)                       |
| rs2278294 | 23.18           | 89.25           | 55.17                         | 67.08                         |
| 95% CI    | (13–34)         | (82–94)         | (35–73)                       | (59–74)                       |

CI, confidence interval; IMPDH, inosine 5'-monophosphate dehydrogenase; MPA, mycophenolic acid. <sup>a</sup>Mean value and 95% CI.

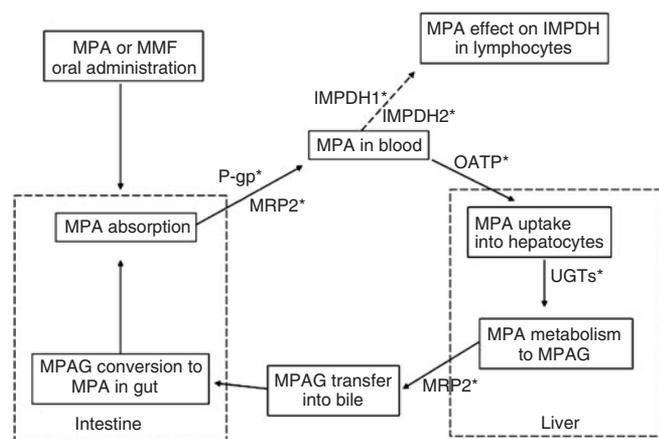
This area contains the entire machinery for enzyme catalytic activity, flanked by a subdomain composed of two regions similar to the cystathionine  $\beta$ -synthase gene (CBS domain) (Figure S2). The subdomain of IMPDH can bind with nucleic acid with nanomolar affinity, but the function of this DNA-binding property is yet to be determined. Our novel

non-synonymous SNPs encode for G519R and D301N, which are both located at the flanking subdomain, and the *in vitro* enzyme kinetic assay and filter-binding assay indicated that the functional consequences of these variants are minor.

A novel *IMPDH1* gene mutation (Arg231Pro) is reported to be associated with a severe form of autosomal-dominant



**Figure 1** LD patterns and haplotype blocks in kidney transplant patients were defined according to the “solid spine of LD” setting in Haploview 3.2 software, which is under the analysis criteria solid spine of LD > 0.8. A standard color scheme is used to display LD pattern, with dark gray for very strong LD (log of likelihood odds ratio, logarithm of odds (LOD)  $\geq 2$ ,  $D' = 1$ ), white for no LD (LOD < 2,  $D' < 1$ ), and bright gray and shades of gray for intermediate LD (LOD  $\geq 2$ ,  $D' < 1$ ); increasing intensity of gray indicates increasing degrees of LD.



**Figure 2** A graphic presentation of the involvement of multiple enzymes and transporters affecting the action of MPA on lymphocyte proliferation. Each of the polymorphisms designated by an asterisk may affect the plasma concentrations of MPA either directly or through the enterohepatic recycling of mycophenolic acid glucuronide (MPAG), with the exception of IMPDH. IMPDH is the target of MPA in *de novo* purine synthesis in lymphocytes.

retinitis pigmentosa. Five different *IMPDH1* variants, Thr116Met, Asp226Asn, Val268Ile, Gly324Asp, and His372-Pro, have been identified in eight autosomal-dominant retinitis pigmentosa families, but their functional significance remains to be defined.<sup>18,19,34</sup> Mutations in *IMPDH1* account

for approximately 2% of families with autosomal-dominant retinitis pigmentosa, and *de novo IMPDH1* mutations are also rare causes of isolated Leber congenital amaurosis.<sup>35</sup> Two *IMPDH1* variants, Arg105Trp and Asn198Lys, were reported in two patients with isolated Leber congenital amaurosis. The Asp226Asn mutation is associated with a severe early-onset form of retinal degeneration. Retinitis pigmentosa is a rare disease, and we did not observe these variations in our transplant patients. The only previous report relating to transplant patients was that a 9-bp insertion within the *IMPDH1* promoter region was found in a patient exhibiting severe azathioprine resistance, possibly by reducing the promoter activity.<sup>36</sup>

Hispanic renal transplant patients are considered to be high-risk patients and have a higher rate of graft failure based on United Network for Organ Sharing (UNOS) data than do the Caucasian renal transplant patients.<sup>37</sup> The rates of biopsy-proven acute rejection have varied in high-risk renal transplant populations given antibody-induction therapy from 15.6%<sup>38</sup> to 26.4%<sup>39</sup> but are as low as 5% at individual centers.<sup>40</sup> No evidence could be found in this study that the inclusion of a significant number of Hispanic patients influenced our analysis of *IMPDH1* polymorphisms and biopsy-proven acute renal transplant rejection.

The accurate assessment of those transplant patients who are at risk for the development of acute rejection will require looking at a multiplicity of immune and pharmacologic factors. In this population of renal transplant patients, *IMPDH1* SNPs were prognostic of the development of biopsy-proven acute rejection in the first postoperative year. The mechanism of association between *IMPDH1* polymorphisms and acute rejection is still to be determined. One possible explanation is their linkage to other SNPs that may control *IMPDH1* mRNA expression, enzyme activity, and ultimately lymphocyte proliferation. Future prospective studies should examine the mechanism by which *IMPDH1* polymorphisms might affect the immunologic response in transplant patients receiving MPA. Both pharmacogenomic and immunogenetic factors will have to be considered in developing treatment algorithms for transplant patients.<sup>41</sup> This study supports the inclusion of *IMPDH1* polymorphisms as one factor that must be tested in developing a panel of gene polymorphisms used to assess the risk of acute rejection for transplant patients being treated with MPA.

## METHODS

**Patient population.** In the *IMPDH*-sequencing initial study, 30 DNA samples of liver transplant patients at the University of Southern California were screened to identify SNP candidates in *IMPDH1*. In the following study, we genotyped 191 kidney transplant patients from the National Institute of Transplantation (St Vincent's Medical Center) in Los Angeles. All kidney transplant patients had been treated from the time of transplantation with tacrolimus, MMF, and prednisone-based immunosuppression, with the exception of five patients who received cyclosporine in place of tacrolimus. The therapeutic serum target level was 5–15 mg/ml for tacrolimus and 100–200 mg/ml for cyclosporine. MMF doses were adjusted based upon patient's tolerance of the adverse effects of the

drug with respect to gastrointestinal side effects and development of leukopenia. The following inclusion and exclusion criteria were used for the renal transplant study: (1) patients who underwent renal transplantation from 2000 to 2005, with available DNA, and had post-transplant survival more than 1 year with follow-up information available; (2) patients must have been on MMF for at least 1 year; and (3) patients must not have been receiving antiviral (valganciclovir, ganciclovir), anticancer, or other leukopenia-causing medications at the time when leukopenia was recorded.

The protocols were approved by the institutional review boards of the University of Southern California and St Vincent's Medical Center in Los Angeles. All patients or guardians provided informed written consent to undergo genotyping. Anticoagulated venous blood was obtained from each patient, and DNA was extracted from whole blood using a commercially available DNA extraction procedure (Qiagen, Valencia, CA). Sequencing and genotyping were performed in patients who had been previously transplanted, and all patient information was collected retrospectively. Patients were managed according to the standard transplant center protocol, which did not include monitoring of MPA plasma concentrations. At most transplant centers, MPA doses are adjusted based upon patient's tolerance of the adverse effects of the drug with respect to gastrointestinal side effects and the development of leukopenia.

**Genotyping.** SNP identification in the *IMPDH1* gene was performed by PCR amplification followed by bidirectional direct DNA sequencing, as described previously.<sup>27</sup> PCR was carried out in a total volume of 50  $\mu$ l using 50 ng of genomic DNA, 5 pmol of each forward and reverse primer (Sigma, St Louis, MO), 0.2 mM dNTP (Promega, Madison, WI), 1  $\times$  PCR buffer, and 1.5 U of thermostable *Taq* DNA polymerase and *Tgo* DNA polymerase with proofreading activity and high fidelity (Expand 20 kb<sup>PLUS</sup> PCR System; Roche Applied Science, Penzberg, Germany). The primers used are described in **Table S1**. The PCR process included initial denaturation at 92°C for 2 min and 10 cycles of denaturation at 92°C for 10 s, annealing at 57°C for 30 s, and synthesis at 68°C for 7 min followed by additional 25 cycles of denaturation at 92°C for 10 s, annealing at 57°C for 30 s, and synthesis at 68°C for 7 min, with the time extended by 10 s for each successive cycle. The final elongation was carried out for 5 min at 72°C. Amplified PCR products were purified by using the Qiagen PCR purification kit (Qiagen) and sequenced by conventional means using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on ABI 377 XL Sequencer (Applied Biosystems, Foster City, CA). SNPs were identified by transferring the chromatograms to sequence assembly software Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI). Each base call was compared with the consensus sequence, and the SNPs were confirmed by visual inspection of the chromatograms.

For genotyping the SNPs identified, we genotyped 191 transplant patients by TaqMan assay using the ABI 7900 Real-Time PCR System (Applied Biosystems). Two oligonucleotide probes for each SNP were synthesized by Applied Biosystems. DNA concentration was measured photometrically and the DNA was diluted to a concentration of 2 ng/ $\mu$ l. Amplification was performed in a final volume of 25  $\mu$ l DNA solution at a concentration of 2 ng/ $\mu$ l, 100 pmol/ $\mu$ l of each probe, 2.5  $\mu$ l of TaqMan Universal PCR Master Mix (Applied Biosystems), and distilled water. In every assay, controls for the wild type and mutations were included. Reaction mixtures were loaded into 96-well plates and placed in an ABI Prism Sequence Detector 7900 (Applied Biosystems). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (94°C for 15 s), annealing, and extension in one step (60°C for 60 s).

A representative chromatogram of the (a) G519R and (b) D301N heterozygotes for the *IMPDH1* gene is shown in **Figure S1**. The arrows indicate that the variant is C/T and A/G in the sense sequence (A, adenine; G, guanine; C, cytosine; and T, thymine).

**Statistical analysis.** Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium using Arlequin version 3 (<http://cmpg.unibe.ch/software/arlequin3>). Determination of linkage disequilibrium (LD), tagging SNPs, haplotype blocks, and frequencies were performed by using Haploview version 3.2. Haplotype blocks were defined according to the "solid spine of LD" setting in Haploview 3.2 software, which is under the analysis criteria solid spine of LD > 0.8.

Each variant in *IMPDH1* underwent univariate analysis for the difference of allele distribution between patients with or without leukopenia and between patients with or without rejection within 1-year post-transplantation by  $\chi^2$  test. Considering the low frequency of the minor alleles, we combined the homozygous variant with the heterozygous patients. Therefore, the presence or absence of the variant alleles, instead of genotype, was compared in this population. Multiple logistic regression analysis was performed to examine the association between *IMPDH1* variants and acute rejection in patients. For SNPs with *P*-value < 0.05, clinical prognostic values (sensitivities, specificities, negative and positive predictive values) were assessed for their association with acute rejections occurring in the first postoperative year. All *P*-values < 0.05 were considered as significant. Statistical analysis was performed using the SPSS software program version 14.0 for Windows (SPSS, Chicago, IL).

The incidence of both leukopenia and rejection was treated as a dichotomous trait. Patients with a white blood cell count > 3,000/ml were considered as not having leukopenia, and patients with a white blood cell  $\leq$  3,000/ $\mu$ l during MPA treatment were considered as having leukopenia. Further subgroup analysis was carried out using the grades of leukopenia described by the Southwest Oncology Group (SWOG).<sup>42</sup>

**SUPPLEMENTARY INFORMATION** is linked to the online version of the paper at [www.cptjournal.org](http://www.cptjournal.org)

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#### CONFLICT OF INTEREST

The authors declared no conflict of interest.

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