Long-Term Results of TPMT Activity Monitoring in Azathioprine-Treated Renal Allograft Recipients

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Abstract. Thiopurine methyltransferase (TPMT) is implicated in the metabolism of azathioprine. The consequences of differential TPMT activity induction by azathioprine on the long-term results after renal transplantation were investigated. The erythrocyte TPMT activity in 82 patients on days 0, 7, and 30 was prospectively evaluated. Because various patterns of TPMT activity variation were noted, the population was subsequently divided between inductors (n = 47) and noninducers (n = 35). Data regarding patient and graft survival and acute rejection episodes were collected. Renal allograft assessment was performed at 3 mo and 2 yr to evaluate the renal function and the histologic lesions on routine biopsies. Data regarding azathioprine-related toxicity also were collected. In a subgroup of patients (n = 19), azathioprine blood levels were determined at day 7 and day 30. The graft survival censoring death was statistically improved in TPMT inductor patients when compared with non-TPMT inductors (P < 0.05). Among TPMT inductors, an acute rejection episode was observed in 34% of the patients versus 69% among non-TPMT inductors (P = 0.002). At 3 mo, serum creatinine was significantly lower among TPMT inductors when compared with non-TPMT inductors (123.1 ± 7.6 and 161.4 ± 13.9 μmol/L, respectively; P = 0.01). On routine allograft biopsies at 2 yr (n = 61), grade 2 or 3 chronic lesions were present in 19% versus 25%, respectively (P = NS). At days 7 and 30, the azathioprine blood levels were higher among patients who experienced acute rejection (P < 0.02). TPMT activity induction was observed in 57% of renal transplant recipients who received azathioprine. This induction was associated with better graft outcome. The appropriate conversion from azathioprine, which is a pro-drug, into 6-mercaptopurine could explain both better graft outcome and TPMT induction. Assessing the ability of azathioprine metabolism at an individualized level before transplantation may allow a more accurate choice among the different immunosuppressive treatments.

Genetic polymorphism in drug metabolism may influence the optimal dose of medication for each individual patient or the most appropriate medication for a given disease. After renal transplantation, the choice between different immunosuppressive drugs is now possible. Among purine synthesis inhibitors, one may consider using either azathioprine or mycophenolate mofetil. Although azathioprine has been used extensively for almost 30 yr in kidney transplantation and autoimmune diseases (1–4), its metabolism, which accounts for both its immunosuppressive efficacy and its toxicity, was understood only recently. Schematically, azathioprine is converted mainly in the liver into 6-mercaptopurine (6-MP), possibly as a result of a glutathione-S-transferase (GST) catalyzed reaction (5). Further conversion of 6-MP occurs in the liver and the gut (6). Three major pathways are important in 6-MP metabolism.

Conversion of 6-MP by the enzyme hypoxanthine guanine phosphoribosyltransferase leads to the formation of 6-thioguanine-nucleotides (6-TGN) (6), which act as metabolic analogs and are responsible for both the immunosuppressive activity and the toxicity of azathioprine (7). The thiopurine methyltransferase (TPMT) pathway leads to the methylation of 6-MP forming 6-methylmercaptopurine (6-MMP). Finally, conversion by the enzyme xanthine oxydase leads to the formation of 6-thiouric acid (Figure 1).

TPMT activity in humans, which is inherited as an autosomal-codominant trait, exhibits genetic polymorphism. Approximately 10% of the Caucasian population inherits intermediate activity and 0.03% inherits TPMT deficiency (8). The cloning of the TPMT gene and the recent identification of inactivating mutations in the human TPMT gene has enlightened the genetic basis for the TPMT polymorphism (9–12).

Several papers reported the correlation between TPMT activity and the efficacy and toxicity of both azathioprine and mercaptopurine treatments (13–17). We previously reported in a small population of renal transplant recipients that the induction of TPMT activity is induced by azathioprine treatment during the first month after transplantation (18). This inducibility of TPMT activity was associated with better short-term
outcome after transplantation. The aim of the present study was to evaluate the long-term impact of the TPMT inducibility in a larger renal transplant recipient population.

Materials and Methods
Study Population
Between 1991 and 1996, we prospectively included 132 renal transplant recipients who were receiving an azathioprine-based immunosuppressive regimen. We excluded from the analysis 19 patients because of blood transfusion during the first month before and after transplantation because erythrocyte transfusions interfere with TPMT activity. Thirty-one patients were excluded because of inadequate blood samples. The final population available for analysis was 82 patients. Some of these patients were included in a preliminary study on short-term consequences of TPMT activity reported elsewhere (18). The demographic characteristics of the study population are shown in Table 1.

Immunosuppressive Treatment
The initial immunosuppressive regimen associated steroids (initial dosage of 1 mg/kg per d tapered to 0.25 mg/kg per d at the end of the first month) and azathioprine (2 to 3 mg/kg per d). Cyclosporine was given to all patients except 18 at an initial dosage of 6 mg/kg per d adapted to trough level ranging from 150 to 250 ng/ml (whole blood, TDx monoclonal antibody polarization immunoassay). Cyclosporine was started at day 0 in 4 patients, at day 8 in 55 patients, and at day 20 in 5 patients. Seventy-nine patients received an induction immunosuppressive regimen including either polyclonal antibodies (Thymoglobulin, Mérieux Institute, Lyon, France; n = 47) or an anti-CD3 monoclonal antibody (OKT3; n = 26) or anti-LFA1 monoclonal antibody (n = 6). Biopsy-proven rejection episodes were treated with high-dose steroids for the first episode and, for subsequent episodes, with polyclonal antibodies or OKT3 for 10 d.

Efficacy and Safety Follow-Up
At 3 mo and 2 yr after transplantation, serum creatinine and data regarding azathioprine side effects, i.e., liver function tests, total leukocytes, and polymorphonuclear leukocytes count, were collected. A routine biopsy was performed at 3 mo and 2 yr. These biopsies were analyzed using the 1997 Banff classification (19) and reviewed blindly by a trained pathologist (L.H.N.).

TPMT and Azathioprine Levels
TPMT activity was measured at days 0, 7, and 30 in erythrocyte cytosol using a radiochemical method (20) as described. Briefly, 6-MP was converted in vitro to 6-MMP in the presence of S-adenosylmethionine (SAM). The methyl group was labeled with 14C. Five ml of blood was collected on heparin. Cytosol of red blood cells was prepared and stored if necessary at -280°C (no variation in TPMT activity was observed after storage). On the day of TPMT activity measurement, 900 μl of red blood cell cytosol was treated with 100 μl of Chelex 100 and gently rotated for 1 h at 4°C. The assay was performed as follows: 25 μl of potassium phosphate buffer, 150 mM (pH 7.5) was added to 100 μl of the lysate followed by the addition of 5 μl of 6-MP (18 mg/ml) in dimethyl sulfoxide. The reaction was initiated by the addition of 25 μl of the following reagent mixture (final concentration in 150 μl indicated): 14C-methyl-labeled SAM,

Table 1. Donors’ and recipients’ demographic characteristics in the whole population and according to the TPMT inductor group

<table>
<thead>
<tr>
<th></th>
<th>All Patients Evaluable</th>
<th>TPMT Inductors (group 1, n = 47)</th>
<th>Non-TPMT Inductors (group 2, n = 35)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age (yr)</td>
<td>35.4 ± 1.3</td>
<td>34.7 ± 1.9</td>
<td>36.3 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Donor gender (M/F)</td>
<td>51/31</td>
<td>30/17</td>
<td>21/14</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient age (yr)</td>
<td>40.4 ± 1.5</td>
<td>41.3 ± 1.8</td>
<td>39.1 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient gender (M/F)</td>
<td>52/30</td>
<td>12/8</td>
<td>40/22</td>
<td>NS</td>
</tr>
<tr>
<td>Transplant rank (first transplant)</td>
<td>64</td>
<td>39</td>
<td>25</td>
<td>NS</td>
</tr>
<tr>
<td>Cold ischemia time (h)</td>
<td>22.2 ± 0.9</td>
<td>22 ± 1.1</td>
<td>22.5 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>HLA MM total</td>
<td>2.21 ± 0.14</td>
<td>2.13 ± 0.17</td>
<td>2.31 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>HLA MM Dr</td>
<td>0.61 ± 0.07</td>
<td>0.53 ± 0.08</td>
<td>0.71 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Delayed graft function (%)</td>
<td>24.3</td>
<td>17.5</td>
<td>59.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Azathioprine initial dose (mg/kg)</td>
<td>2.32 ± 0.07</td>
<td>2.28 ± 0.13</td>
<td>2.28 ± 0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Steroid initial dose (mg/kg)</td>
<td>0.95 ± 0.03</td>
<td>0.97 ± 0.02</td>
<td>0.92 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

* TPMT, thiopurine methyltransferase; HLA MM, human leukocyte antigen mismatch.
12.5 × 10⁻⁶ M, nonradioactive SAM, 12.5 × 10⁻⁶ M. Dithiothreitol 10⁻³ to stabilize 6-MP and allopurinol to inhibit xanthine oxidase were added to the incubation medium. Each tube was incubated for 1 h at 37°C in a shaking water bath, and the reaction was stopped by the addition of 500 μl of borate buffer, 0.5 M, pH 10. The whole volume was transferred to a glass conical centrifuge tube containing 5 ml of 20% isomyl alcohol in toluene. The tube was stoppered and mixed vigorously for 10 s. After centrifugation at 700 g for 10 min, 2 ml of the organic phase was removed and placed in a liquid scintillation-counting vial containing 2 ml of scintillation liquid. Radioactivity was measured in an LKB liquid scintillation counter. The blank tubes included all reagents except 6-MP. All results were corrected for the extraction of 6-MMP into the organic phase (44%) and for counting efficiency. TPMT activity was expressed as nanomoles of 6-MMP formed per hour and per milliliter of packed red blood cells. Control incubations were performed without substrate. The reagents were of analytical grade form Sigma (St Louis, MO), Prolabo (Paris, France), or Boehringer-Mannheim (Mannheim, Germany).

Control incubations were performed without substrate. The reagents were of analytical grade form Sigma (St Louis, MO), Prolabo (Paris, France), or Boehringer-Mannheim (Mannheim, Germany). SAM, with its methyl group labeled by 14C (59 Ci/mol), was purchased from Amersham (Buckinghamshire, England), and Chelex 100 was purchased from Bio-Rad (Richmond, CA).

In addition, azathioprine dosage was performed using high-performance liquid chromatography technique in 19 patients. Extraction of plasma samples was performed as described using a radial compression liquid chromatography and silica solid phase extraction (21). Plasma samples were extracted by use of Sep-Pak silica cartridges (1 ml volume; Water Associates, Milford, MA). After the cartridge was pretreated with 3 ml ethyl acetate and vacuum-dried using a Sep-Pak Cartridge Rack (Waters Associates) for 1 min, the plasma sample (1 ml) associated with 6 μM antipyrine as internal standard was introduced into the cartridge and washed by passing 5 ml of hexane over 1 min. The cartridge was vacuum-dried again for 1 min, and the compounds were eluted with 5 ml of ethyl acetate. The eluate was brought to dryness under a gentle stream of nitrogen gas, and the residue was reconstituted with 200 μl of the mobile phase by ultrasonication. The solution was transferred to an autosampler microvial and injected in the chromatograph. The separation of the drug and internal standard (antipyrine 6 μM) was achieved on a C18 cartridge.

Statistical Analyses

Because TPMT activity is not normally (Gaussian) distributed, nonparametric statistical tests were used for analysis of enzyme activities. TPMT is thus expressed as the median and range. We used nonparametric statistical tests (the Mann-Whitney U test for comparison between two groups and the Kruskal-Wallis test for comparison among several groups). The other parameters (drug, dose, age, etc.) after confirmation of normal distribution were expressed as mean ± SEM, and both t test and ANOVA were used for comparison between groups. Correlation within a group was expressed by the Spearman rank correlation coefficient. In this prospective study, the χ² test and the contingency table were used for comparison of subgroups of patients. Differences in actuarial (Kaplan-Meier) graft and patient survival were tested by the log-rank method. Probability values less than 0.05 were accepted as statistically significant.

Results

TPMT Activity

TPMT erythrocyte activity rose after renal transplantation from day 0 to day 7 and day 30 (P < 0.0001). As shown in Table 2, patients could be further subdivided into three groups according to the different patterns of modification of TPMT activity. The definition of TPMT activity induction was a more than 10% increase of the initial value. This cutoff was determined according to the intraindividual variation (data not shown). This increase was observed as early as day 7 after transplantation in 22 patients and at 1 mo after transplantation in 25 patients, whereas TPMT activity remained stable in 35 patients (Figure 2). Demographic and treatment characteristics of each group are presented in Table 1.

![Figure 2. Evolution of TPMT activity over time.](image)
Biopsy-Proven Acute Rejection

A total of 42 acute rejection episodes were observed in the whole population. Among TPMT inductors, 16 of 47 patients (34%) experienced at least one acute rejection episode. Conversely, in non-TPMT inductors, 24 of 35 patients (69%) experienced at least one acute rejection episode. This difference was significantly different between the two groups (P = 0.002). The first acute rejection episode was also delayed in the TPMT inductor group when compared with non-TPMT inductor group (median [range], 36 [6 to 170] d versus 26.5 [8 to 74] d, respectively; Figure 3).

Patient and Graft Survival

Patient survival and graft survival with and without censoring death were analyzed. At 1 yr, the graft survival censoring death was 91% in TPMT inductors versus 77% in non-TPMT inductors. At the end of the follow-up period, it was statistically improved (87% versus 60%, P < 0.05; Figure 4). The patient survival in the TPMT induction group was 96% versus 76%. The graft survival was 83% versus 54%, respectively. This was not statistically different, however.

In the TPMT induction group, patient survival at 1 yr and at the end of the follow-up period was 100% and 95.6% versus 94.3% and 76.4%, respectively, in the non-TPMT induction group (P = NS). In the TPMT induction group, graft survival at 1 yr and at the end of the follow-up period was 91.5% and 82.9% versus 77.1% and 54.4%, respectively, in the non-TPMT induction group (P = NS).

Renal Graft Function and Histology

Renal graft function was appreciated by serum creatinine at 3 mo and 2 yr after transplantation. At 3 mo, renal function was significantly better among TPMT inductors versus non-TPMT inductors (123.1 ± 7.6 and 161.4 ± 13.9 μmol/L, respectively, P = 0.01). This difference was not significant at 2 yr (117.6 ± 6.6 versus129.1 ± 9.4 μmol/L, respectively).

Histologic evaluation was performed on routine biopsies at 3 mo (n = 68) and 2 yr (n = 61) after transplantation (Table 3). At 3 mo, acute lesions were less frequent in TPMT inductors (7 biopsies among 40 [20%]) versus non-TPMT inductors (5 biopsies among 28 [32%]). Chronic lesions were already present in 8 (20%) versus 10 biopsies (36%), respectively (P = NS).

At 2 yr, almost no acute rejection lesion was observed in either group. Chronic lesions were present in 49% of the biopsies performed in TPMT inductors versus 55% in non-TPMT inductors. Grade 2 or 3 chronic lesions were diagnosed in 19% versus 25%, respectively. These differences did not reach statistical significance.

Safety Analysis

There was no statistically significant difference between the two groups with regard to liver enzyme levels or bilirubin dosage.

There was no difference in total white blood cell counts. From day 15, a significant decrease in polymorphonuclear leukocyte count was observed in non-TPMT inductors when compared with TPMT inductors (7610 ± 494 versus 5778 ± 644, respectively; P < 0.02). This difference was still present at 2 yr (3552 ± 290 versus 2320 ± 296, respectively; P < 0.01).

Azathioprine Level

To explore further the mechanism of this differential induction of TPMT activity and the possible decreased level of azathioprine transformation into 6-MP in the non-TPMT inductors, we performed azathioprine dosage at day 7 and day 30 in a subset of patients (n = 19). At days 7 and 30, the
azathioprine blood trough level was 2.9 ± 0.3 and 2.9 ± 0.4, respectively, in the TPMT inductor groups versus 4 ± 0.5 and 3.9 ± 0.4, respectively, in the non-TPMT inductor group (P = NS). Azathioprine levels at day 7 and day 30 were significantly higher in the group of patients with an acute rejection episode (4.3 ± 0.5 and 4.2 ± 0.3, respectively) when compared with the group of patients without acute rejection (2.6 ± 0.3 and 2.5 ± 0.3, respectively; P < 0.02).

Discussion

TPMT activity polymorphism is an interesting paradigm in the field of pharmacogenetics. It has been demonstrated that azathioprine and mercaptopurine toxicity may be more frequent in genetically TPMT-deficient patients (13–16). Phenotypic characterization and molecular genetic methods have been developed to diagnose these patients (22,23). However, despite various attempts to obtain a simple and accurate monitoring of these drugs, the results remain contradictory. We already reported that the use of azathioprine after renal transplantation is associated with a TPMT induction (18). The results of this larger prospective study confirm our findings. Furthermore, TPMT induction has been observed in patients with leukemia treated by mercaptopurine (W. E. Evans, personal communication, November 1999).

TPMT induction is associated with a lower incidence of clinical acute rejection. It has been reported that in children who had acute lymphoblastic leukemia and were treated with mercaptopurine, the outcome was significantly worse in patients with higher TPMT activity (16). Chocair et al. (17) suggested that the same phenomenon occurs after renal transplantation. It is thought that a higher TPMT activity should decrease 6-TGN concentrations. 6-TGN are the active and toxic products. Our results show that in most patients, efficacy is not related to the baseline TPMT activity but to the induction of this activity after azathioprine treatment. In our patients, neither baseline TPMT activity nor day 30 TPMT activity correlated with graft and patient outcome (data not shown). We also observed a significant improvement of long-term graft survival censoring death. Death is an important cause of late graft loss, but we wanted to evaluate the efficacy of azathioprine in different subpopulations. It can be assumed that the graft survival censoring death is a more accurate end point to evaluate efficacy of an immunosuppressive treatment. There was also a trend for better renal allograft histology at 2 yr even though the difference was not statistically significant. This long-term improvement may be secondary to the better prevention of acute rejection episodes during the first 3 mo. However, the direct relationship between allograft long-term outcome and the incidence of early acute rejection episode is questionable since the results of long-term follow-up of the newer immunosuppressive drugs pivotal trials. Indeed, in these trials, the use of neither tacrolimus (24) nor mycophenolate mofetil (25,26) was associated with long-term improvement of renal graft survival despite an initial improvement in the incidence of acute rejection episodes. Another explanation for the positive impact of TPMT induction on long-term results may be that TPMT higher activity is still present several years after transplantation.

TPMT activity induction may be secondary to various azathioprine metabolism. Azathioprine is converted into 6-MP. If azathioprine is only poorly metabolized, 6-MP levels will be low and 6-MP will not induce TPMT activity. On the contrary, if azathioprine is well converted into 6-MP, then 6-MP levels will be high and TPMT activity will be induced (Figure 1). Therefore, TPMT activity may be induced by the rise of the concentration of its substrate, 6-MP. Unfortunately, 6-MP concentrations are difficult to assess during a clinical study. In accordance with this explanation, we found in a small subset of patients a higher concentration of azathioprine in non-TPMT inductor patients when compared with TPMT inductor patients because azathioprine is not correctly converted into 6-MP. Azathioprine levels were also significantly higher in the group of patients who experienced clinical acute rejection episode. This preliminary findings need to be confirmed. These results are not contradictory with Bergan et al.’s (7) report, which demonstrated that higher azathioprine dosage is associated with higher 6-TGN levels and a reduction of acute rejection rate. If azathioprine is converted into 6-MP, then azathioprine blood levels may be low in these patients as well. In our study, we report a negative impact of high azathioprine blood levels because it reflects the absence of proper metabolism.

The metabolism of azathioprine is not fully understood. It has been reported that GST is implicated in the first enzymatic step of azathioprine liver metabolism (6). In vitro inhibition by ethacrynic acid of GST-related azathioprine metabolism in a liver microsome model supports this hypothesis (personal

### Table 3. Histologic evaluation at 3 mo and 2 yr of renal allograft biopsies according to the inductor groups

<table>
<thead>
<tr>
<th></th>
<th>Acute Lesions</th>
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<th>Chronic Lesions</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Grade 0</td>
<td>Grade 1</td>
<td>Grade 2</td>
<td>Grade 3</td>
</tr>
<tr>
<td>3 mo (N = 68)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inductors (n = 40)</td>
<td>80%</td>
<td>7.5%</td>
<td>7.5%</td>
<td>5%</td>
</tr>
<tr>
<td>noninductors (n = 28)</td>
<td>68%</td>
<td>14%</td>
<td>14%</td>
<td>4%</td>
</tr>
<tr>
<td>2 yr (N = 61)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inductors (n = 37)</td>
<td>92%</td>
<td>5%</td>
<td>3%</td>
<td>0%</td>
</tr>
<tr>
<td>noninductors (n = 24)</td>
<td>87.5%</td>
<td>12.5%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Results are presented according to the Banff 1997 classification for acute and chronic lesions.
data). We have already reported that GSTM1-1 polymorphism is not involved in the metabolism of azathioprine (18). We are undergoing evaluation of other isoenzymes, such as GSTT1. GSTT1 exhibits genetic polymorphism, and the null mutant recently has been associated with a higher incidence of acute rejection after transplantation, which is consistent with the hypothesis (27). Finally, it might be possible to develop a phenotypic test of azathioprine metabolism after incubation with erythrocytes (personal data). Using these genotypic, i.e., GSTT1 polymorphism assessment, and phenotypic, i.e., azathioprine erythrocyte metabolism, tests before transplantation may help to choose between purine synthesis inhibitor, namely azathioprine or mycophenolate mofetil. Furthermore, azathioprine-mycophenolate mofetil conversion has been tried after organ transplantation to avoid long-term overimmunosuppression and overcost. However, this maneuver carries the risk of acute rejection episode (28). This risk may be higher in patients who are unable to metabolize azathioprine efficiently. The in vitro phenotypic and genotypic testing could allow individualization of long-term immunosuppressive treatment.

In conclusion, the inducibility of erythrocyte TPMT activity is related to better short- and long-term results after renal transplantation. This improvement may be secondary to the better ability of azathioprine to be metabolized into 6-MP in some patients. The pre- and posttransplantation determination of this metabolism may allow an individualized strategy for immunosuppressive use. Furthermore, we are currently evaluating the presence and significance of TPMT activity induction in patients who are receiving azathioprine treatment for autoimmune diseases.

References


