

An Exploratory Study on Pharmacogenetics of Inosine-Monophosphate Dehydrogenase II in Peripheral Mononuclear Cells From Liver-Transplant Recipients

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ABSTRACT

Mycophenolate mofetil (MMF) is an immunosuppressant used for the prophylaxis of rejection in renal, pancreas, and liver transplantation. It inhibits the inducible isoform of the enzyme inosine-monophosphate dehydrogenase (IMPDH II) via its active metabolite mycophenolic acid (MPA). IMPDH II is necessary for de novo purine synthesis in activated lymphocytes. The aims of the present study were to evaluate the feasibility of a real-time polymerase chain reaction (PCR) quantitative assessment of IMPDH II gene expression in liver transplant recipients as well as to provide a preliminary evaluation of possible correlations with drug tolerability. RNA was extracted from peripheral blood mononuclear cells of liver recipients after at least 6 months of MMF administration. IMPDH II gene expression was assessed using quantitative, real-time PCR and normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Finally, adverse events associated with MMF administration were recorded. Real-time PCR quantitation of IMPDH II gene expression was reliable, sensitive, and specific. The intrapatient variability for both IMPDH II and GAPDH assays was lower than 0.6% in all patients. The results demonstrated a wide interpatient variability, with the mean value \pm standard deviation of 0.949 ± 0.525 (95% confidence interval, 0.669–1.229) and a median value of 0.797. Patients with treatment-related toxicities displayed a trend to a higher level of IMPDH II expression than those without toxicity (mean, 1.126 vs 0.771). In conclusion, pharmacogenetic analysis of IMPDH II may represent a novel approach to MMF therapeutic monitoring.

IN RECENT years, substantial improvements in graft survival have been obtained owing to the increasing number of effective immunosuppressive drugs. Mycophenolate mofetil (MMF) is an immunosuppressant widely used for the prophylaxis of organ rejection in renal, pancreas, and liver transplantation.^{1,2} It inhibits the inducible isoform of the enzyme inosine-monophosphate dehydrogenase (IMPDH II) via its active metabolite mycophenolic acid (MPA). IMPDH II is necessary for de novo purine synthesis in activated lymphocytes, whereas other cells generate purine nucleotides via the salvage pathway.³ Noteworthy,

IMPDH II is 5 times more sensitive to MPA inhibition than the constitutive isoform.⁴

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Several studies demonstrate a large interpatient variability both in basal IMPDH activity and in time required for a complete recovery of enzyme activity after MMF administration.⁵ These findings emphasize the role of pharmacodynamic monitoring as a tool to optimize drug dosage and schedule of administration, including tacrolimus and cyclosporine;^{6,7} however, available methods for titration of IMPDH activity are complex and time consuming.⁵ Therefore, the aims of the present study were to evaluate the feasibility of a real-time polymerase chain reaction (PCR) quantitation of IMPDH II gene expression in liver transplant recipients and to provide a preliminary evaluation of the correlation with MMF pharmacodynamics as a new approach to treatment optimization.

PATIENTS AND METHODS

Patients

Patients with liver transplants were enrolled in the present study after the study was approved by the local Ethics Committee and all subjects gave informed consent to the genetic analysis. Inclusion criteria required stable MMF therapy for at least 2 months, no changes in the immunosuppressive protocol (drugs and/or dosage), and satisfactory liver and kidney function. All toxicities associated with the treatment were recorded. Patients were divided into 2 groups according to the length of MMF administration at the time of blood collection for gene expression analysis, ie, group A was under therapy for less than a year, and group B for more than a year.

Materials

Lymphoprep Separation Medium was obtained from ICN Bio-medicals (Costa Mesa, Calif, USA), whereas all other chemicals, of the highest purity available, were obtained from Sigma-Fluka-Aldrich (Milan, Italy).

Collection of Lymphocytes

In all patients, 15 mL of blood were drawn before the MMF morning dose at least 6 months after the beginning of drug administration. Blood was kept in heparinized tubes, diluted 1:1 with phosphate-buffered saline (PBS, pH 7.4) and layered over Lymphoprep Separation Medium. After centrifugation for 40 minutes at 1100 rpm, the lymphocyte layer was removed and washed twice with PBS; then contaminating erythrocytes were lysed by diluting the cell suspension 1:8 (v/v) with hypotonic solution (KHCO₃ 10 mmol/L, NH₄Cl 160 mmol/L, EDTA 0.13 mmol/L) for 15 minutes at room temperature. The cell suspension was then centrifuged at 10000 rpm for 15 minutes. The resulting pellet was washed with PBS and finally resuspended in 35 mmol/L sodium phosphate buffer (pH 7.4).

Gene Expression Quantitation

The expression of IMPDH II and of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed using quantitative real-time PCR (QRT-PCR). Briefly, total RNA was extracted from cells using the Qiagen RNeasy MinElute Cleanup kit (Qiagen, Milan, Italy). RNA (0.4 µg) was reverse transcribed at 37°C for 1 hour in the presence of Moloney Murine Leukemia Virus-Reverse Transcriptase (200 U/µL), deoxyribonu-

cleotides triphosphate (10 mmol/L each nucleotide), random ex- amers (500 µg/mL), PCR buffer (Tris-HCl 250 mmol/L, KCl 375 mmol/L, MgCl₂ 15 mmol/L), and ribonuclease inhibitor (40 U/µL) in a final volume of 20 µL. QRT-PCR was performed on comple- mentary DNA (cDNA) samples using the ABI Prism 7900 Se- quence Detection System (Applied Biosystems, Foster City, Calif, USA). Briefly, 5 µL of PCR products were mixed with each primer, probe (100 mmol/L), and 12.5 µL of Taqman Universal PCR Master Mix (Applied Biosystems). Thermal cyclers parameters included 2 minutes at 50°C, 10 minutes at 95°C, and 35 cycles involving denaturation at 95°C for 15 seconds and annealing/ extension at 60°C for 1 minute.

Primers and probe for IMPDH II were as follows: forward; 5'-CACTCATGCCAGGACATTGGT-3'; reverse, 5'-AAACTTA- AGCTCCCAGAGTACATCA-3'; probe, 5'-CTTGACCCA AGTCCGAG-3'. They were designed on the basis of the sequence of IMPDH II gene (Gene Bank, accession number L39210) using the Primer Express 2.0 software (Applied Biosystems). Amplicons were less than 100 bp in length and they spanned 2 adjacent exons, to enhance PCR efficiency, and exclude amplification of contami- nating genomic DNA. IMPDH II probe was labelled with a fluorescent reporter dye (FAM) at the 5' end of nucleotide. Primers and probes for GAPDH expression were purchased from Applied Biosystems.

The quantitation of gene expression was performed using a comparative method after validation experiments; the threshold cycle (C_t) was defined as the number of PCR cycles required to obtain the threshold fluorescence, and its value was normalized to GAPDH (-ΔC_t). Finally, all values were normalized with respect to that obtained in a sample taken as a reference (-ΔΔC_t) and expressed as 2^{-ΔΔC_t}. All samples were analysed in triplicate.

Statistical Analysis

Results were expressed as mean values ± standard deviations (SD). Percentage coefficient of variation was defined as the ratio between standard deviation and the mean. All statistical calculations were performed using GraphPad Prism 4.0 software (GraphPad, San Diego, Calif, USA).

RESULTS

The clinical features of the 16 patients are summarized in Table 1. Liver transplantation was required for end-stage liver disease, mostly viral hepatitis (hepatitis C virus [HCV] and hepatitis B virus [HBV] infection). After surgery, all patients showed satisfactory graft function. Maintenance immunosuppression consisted of MMF (1–2 g/d), in combination with cyclosporine (12 patients) or tacrolimus (4 patients; Table 1).

Real-time PCR assay proved to be reliable; analysis of triplicate measurements showed a coefficient of variation of less than 0.6% for GAPDH and IMPDH II. Mean value ± SD C_t values were 29.16 ± 2.48 and 26.80 ± 2.69 for IMPDH II and GAPDH, respectively.

A wide range in IMPDH II gene expression was observed with a mean value ± SD 0.949 ± 0.525 (95% confidence interval, 0.669–1.229) and a median of 0.797. Stratification of patients on the basis of the duration of MMF adminis- tration less or more than 1 year revealed quite similar IMPDH II expression (0.953 ± 0.558 and 0.945 ± 0.530,

Table 1. Demographic Characteristics of Enrolled Patients

| Patient | Sex/Age (ys) | Hepatic Disease | Length of MMF Therapy (mos) | MMF Daily Dose (g) | Other Immunosuppressive Drugs | IMPDH II Gene Expression | Toxicity/Degree* |
|---------|--------------|---|-----------------------------|--------------------|-------------------------------|--------------------------|-----------------------------------|
| 1 | M/62 | Toxic cirrhosis | 18 | 0.5 × 2 | Cyclosporine (200 mg/d) | 0.783 | None |
| 2 | M/47 | HCV ⁺ cirrhosis | 19 | 1.0 × 2 | Cyclosporine (125 mg/d) | 0.439 | Thrombocytopenia/mild |
| 3 | M/45 | HCC (HBV ⁺ , HCV ⁺ cirrhosis) | 19 | 0.5 × 2 | Cyclosporine (150 mg/d) | 1.000 | None |
| 4 | M/60 | HCV ⁺ cirrhosis | 18 | 1.0 × 2 | Cyclosporine (125 mg/d) | 0.475 | Gastritis/mild |
| 5 | M/62 | Toxic cirrhosis | 48 | 0.5 × 2 | Cyclosporine (125 mg/d) | 0.596 | None |
| 6 | M/44 | Toxic cirrhosis, HBV ⁺ , HCV ⁺ , HDV ⁺ | 13 | 0.5 × 2 | Tacrolimus (2 mg/d) | 0.896 | Gastritis/mild |
| 7 | M/57 | HCV ⁺ cirrhosis | 28 | 0.5 × 2 | Cyclosporine (100 mg/d) | 1.336 | None |
| 8 | M/63 | HCC (HCV ⁺ cirrhosis) | 9 | 1.0 × 2 | Cyclosporine (200 mg/d) | 0.842 | Gastritis, CMV infection/moderate |
| 9 | M/41 | HCV ⁺ infection, hemochromatosis | 6 | 1.0 × 2 | Cyclosporine (175 mg/d) | 0.794 | None |
| 10 | F/53 | Liver cholestasis | 9 | 1.0 × 2 | Cyclosporine (150 mg/d) | 2.112 | Gastritis/mild |
| 11 | F/58 | Primitive biliary cirrhosis | 19 | Suspended | Tacrolimus (9.5 mg/d) | 2.031 | Leuko-thrombocytopenia/moderate |
| 12 | M/62 | HCC (HBV ⁺ cirrhosis) | 2 | 0.5 × 2 | Tacrolimus (5 mg/d) | 0.800 | Leuko-thrombocytopenia/mild |
| 13 | M/54 | Toxic cirrhosis | 12 | 1.0 × 2 | Cyclosporine (125 mg/d) | 0.390 | None |
| 14 | M/60 | HCC (HCV ⁺ cirrhosis) | 9 | 1.0 × 2 | Cyclosporine (150 mg/d) | 0.489 | None |
| 15 | M/65 | HCC (HBV ⁺ cirrhosis) | 9 | 0.5 × 2 | Cyclosporine (200 mg/d) | 1.414 | Epigastric pain/mild |
| 16 | M/47 | Toxic cirrhosis | 12 | 0.5 × 2 | Tacrolimus (5 mg/d) | 0.780 | None |

Abbreviations: M, male; F, female; HCC, hepatocellular carcinoma; HDV, hepatitis D virus; CMV, cytomegalovirus.

*Toxicity degree: mild (well tolerated, causing a minimum degree of malaise without affecting normal activities), moderate (interferes with normal activities), and severe (prevents the subject from performing normal activities).

respectively). IMPDH II: gene expression was higher in patients receiving MMF 1.0 g/d (mean value \pm SD, 1.071 ± 0.449 ; median value, 0.896) than in those prescribed 2.0 g/d (0.792 ± 0.609 ; median, 0.489); however, the difference was not significant.

Half of the patients showed thrombocytopenia ($n = 1$), leukopenia and thrombocytopenia ($n = 2$), and gastritis ($n = 5$). Interestingly, there was a trend toward an increase in IMPDH II gene expression among patients experiencing toxicities (1.126 ± 0.656) compared with those who tolerated the treatment (0.771 ± 0.300). Combined analysis of gene expression against toxicities and MMF dose led to the same conclusion.

DISCUSSION

The need for a well-tolerated immunosuppressive regimen led to the introduction of MMF in liver transplantation,² to reduce the dosage or avoid calcineurin inhibitors and

steroids.⁸ However, MMF administration may be associated with bone marrow and gastrointestinal toxicity, thus requiring therapeutic drug monitoring. Furthermore, it has been demonstrated that MMF administration alters IMPDH activity, particularly the isoform II, which is upregulated in activated lymphocytes.⁹ Unfortunately, quantitation of IMPDH activity, as is currently performed, requires radiolabeled compounds or complex analytical methods.

This study examined IMPDH II gene expression using quantitative real-time PCR in liver graft recipients. The present results provide the first evidence that the quantitation of gene transcription of the IMPDH inducible isoform is feasible and reliable. Due to the wide availability of quantitative PCR techniques in clinical laboratories, this approach may represent an important alternative to enzyme activity measurements based on liquid chromatographic methods. The de novo purine salvage pathway in activated lymphocytes, which involves IMPDH II, plays a critical role

in intracellular guanosine monophosphate (GMP) pools,³ and consequently in the immune response to a grafted organ. Therefore, the capability to discriminate between increased expression of the inducible versus the constitutive isoform appears to be of critical importance. Moreover, a quantitative PCR assay may be used to analyse the drug target, thus reflecting MMF efficacy and tolerability, allowing a personalized immunosuppressive therapy.

Although the principal aim of the study was to investigate the feasibility of the gene expression assay, the present data were analysed according to the time of exposure to MMF and dosage, or treatment tolerability. However, neither the time of exposure to nor the dosage of MMF significantly affected IMPDH gene expression. Further studies involving a larger number of samples are required to clarify this relationship.

One interesting trend emerged from the analysis of gene expression in relation to treatment tolerability. Although it is not yet known whether IMPDH II is expressed in tissues other than activated lymphocytes, gastrointestinal or bone marrow toxicities occur in patients with higher gene expression, whereas low IMPDH II messenger RNA (mRNA) levels were related to better treatment tolerability. These results could suggest that the demand for de novo synthesis of GMP, associated with highest IMPDH II mRNA levels, may be associated with greater susceptibility to enzyme inhibition by MPA. This hypothesis has to be strengthened by further studies, mainly with more patients.

In conclusion, IMPDH II gene expression may be reliably

assessed, thus potentially allowing the investigation of genetic factors influencing MMF efficacy and tolerability, a concept that has promising future applications for optimization of immunosuppression.

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