MDRI Polymorphisms and Response to Azathioprine Therapy in Patients with Crohn’s Disease

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Background: To investigate the contribution of multidrug resistance 1 (MDRI) gene pharmacogenetics (G2677T/A and C3435T) to the efficacy of azathioprine in inducing remission in patients with Crohn’s disease (CD).

Methods: A cohort of 327 unrelated Spanish patients with CD recruited from a single center was studied. All patients were rigorously followed up for at least 2 years (mean time, 11.5 years). A case-control analysis of MDRI G2677T/A and C3435T SNPs and 2 loci haplotypes in 112 steroid-dependent CD patients treated with azathioprine was performed. Patients were classified on the basis of response to azathioprine.

Results: A total 76 patients treated with azathioprine for longer than 3 months were included. Remission was achieved in 42 CD patients (55.3%). A higher frequency of the 2677TT genotype was found in nonresponders than in responders (17.65% versus 7.14%; OR = 2.8; 95% CI: 0.6–12.1; P = 0.11). Nonresponders to azathioprine were found to have a higher frequency of the 3435TT genotype than did CD patients who had achieved clinical remission (17.64% versus 4.76%; OR = 4.3; 95% CI: 0.8–22.8; P = 0.06). The 2677TT/3435TT haplotype was also more abundant in nonresponders (29.4% versus 20.2%), whereas the 2677G/3435C haplotype was more frequent in responders (58.3% versus 47.1%). Lack of response to azathioprine therapy in CD patients was 1.8-fold greater in carriers of the 2677T/3435T haplotype than in carriers of the 2677G/3435C haplotype (OR = 1.8; 95% CI: 0.82–3.9; P = 0.14).

Conclusions: The results of our study indicate higher frequencies of the 2677TT and 3435TT genotypes and the 2677T/3435T haplotype in CD patients who did not respond to azathioprine. Additional replications in independent populations would confirm the real impact of these polymorphisms in response to azathioprine therapy.

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Key Words: MDRI gene, Crohn’s disease, azathioprine

Crohn’s disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract characterized by dysregulated mucosal immune response. The etiopathogenesis of the disease remains poorly understood. Experimental and observational data suggest that intestinal inflammation arises from abnormal immune reactivity to bacterial flora in the intestines of individuals who are genetically susceptible.

Genomewide scans searching for inflammatory bowel disease (IBD) susceptibility loci have provided evidence of linkage to different chromosomal regions, with chromosome 7 repeatedly detected. At present, interest has been focused on the multidrug resistance 1 (MDRI) gene, which spans more than 200 kb on 7q. Several polymorphisms of the MDRI gene have been described, among which the C3435T and G2677T/A genomic variants have received considerable attention. Moreover, allelic variations of the MDRI gene determine extension of ulcerative colitis and severity of CD, as well as susceptibility to IBD. The role of the MDRI gene in IBD has been shown by mdrla knockout mice (mdrla<sup>−/−</sup>) spontaneously developing intestinal inflammation similar to that of human IBD, characterized by dysregulated epithelial cell growth and leukocyte infiltration into the lamina propria of the large intestine.

The human MDRI gene encodes the ATP-dependent membrane efflux transporter P-glycoprotein-1 (PGP), expressed in liver, kidney, intestine, and central nervous system endothelial cells. In the human gastrointestinal tract, PGP is found in high concentrations on the apical surfaces of super-
ficial columnar epithelial cells of the colon and the distal small bowel. PGP expression in the intestinal wall and in proximal tubular cells of the kidney reflects its role in the absorption and excretion of xenobiotics and drugs. The effect of PGP on the pharmacokinetics of drugs is well established.

Not responding to medical therapy is a common indication for surgery in patients with IBD. Approximately half of CD patients require surgery in their lifetimes as a result of poor response to medical therapy. Glucocorticoid dependence and resistance have been reported in approximately one third and one fifth of CD patients, respectively. Reduced drug accumulation is a common mechanism of drug failure. In CD, poor response to corticosteroids has been related to increased expression of PGP. Moreover, PGP might play an important role in the pharmacokinetics and pharmacodynamics of prednisone and budesonide, 2 drugs commonly used to treat CD.

Immunosuppressive therapies (cyclosporine, tacrolimus, and methotrexate) used in CD have been identified as substrates of the drug efflux pump PGP. Moreover, genotype monitoring of the MDR1 gene reliably predicts the optimal dose of tacrolimus and weakly predicts that of cyclosporine in renal transplant recipients.

The most commonly used immunomodulatory agent, azathioprine, has been found to be safe and efficacious in inducing remission of CD, with a response rate of 55% to 70%. The results for predicting responsiveness to azathioprine have been conflicting. Recently, it was observed that overexpression of human PGP in leukemia cells produced the efflux of 6-mercaptopurine (an azathioprine analogue) and resistance to this drug.

Our working hypothesis was that altered MDR1 function in patients with inflammatory bowel disease (IBD) could modify the response to medical therapy. This hypothesis is supported by the results of previous studies, which suggested that MDR1 may be an important target of immunosuppressant therapy. In this regard, we aimed to test whether MDR1 pharmacogenetics (C3435T and G2677T/A) plays a role in the efficacy of azathioprine in inducing remission in Spanish CD patients.

### PATIENTS AND METHODS

**Study Population**

We studied a cohort of 327 white unrelated CD patients who were consecutively recruited at an Inflammatory Bowel Disease unit of a single referral center in Madrid, Spain. CD was diagnosed on the basis of standard clinical, radiologic, endoscopic, and histologic criteria. We analyzed data from 112 steroid-dependent patients treated with azathioprine, who were followed up by physicians from our IBD unit. Only 76 patients treated with the drug for longer than 3 months were considered for inclusion. Steroid dependence was defined as recurrent flare-up on reduction or withdrawal of glucocorticoids or as the clinical need for glucocorticoid treatment twice within 6 months or 3 times within a year. Remission in steroid-dependent CD patients was defined as not needing oral steroids (either prednisolone or budesonide) for at least 3 months and having a Harvey-Bradshaw score of 4 or less and in patients with fistulizing CD as complete perianal fistula closure. All patients who did well on low doses of steroids, those needing a surgical procedure, anti-TNF agents, or reintroduction of steroids in less than 3 months while azathioprine was continued, were reported as “remission not achieved.” Criteria for exclusion were: being on azathioprine to prevent postoperative recurrence of CD, having steroid-resistant CD, and having too short a follow-up (<3 months).

Phenotypic details were obtained by both clinical chart review and patient interview. The same clinical questionnaire was completed for each patient. This questionnaire included items on sex, familial IBD, age at diagnosis, follow-up interval, smoking habits, age at diagnosis (as defined by the Vienna classification): A1, < 40 years; A2, ≥ 40 years), disease location (L1, terminal ileum; L2, colon; L3, ileocolon; L4, upper gastrointestinal) and behavior (B1, nonstrictureing nonpenetrating; B2, structuring; B3, penetrating), extraintestinal clinical manifestations (articular, cutaneous, ocular, hepatic), indication for azathioprine, daily dose, toxicity, and date of clinical remission while on the study drug. Clinical data were recorded by a gastroenterologist from the IBD unit (J.L.M.) who was blinded to patient genotype. The study was approved by the Hospital Clinico San Carlos ethics committee.

**Genotyping of MDR1 Polymorphisms**

The MDR1 C3435T polymorphism was genotyped by an Assay on Demand (Applied Biosystems, Foster City, CA, Accession number: C_7586657_1) following the manufacturer’s recommendations. The MDR1 G2677T/A variant was typed by allele-specific PCR (sense: 5'-GAT AAG AAA GAA CTA GAA GGT G/T/A-3'; antisense: 5'-TCA ATC ATA TTT AGT TTG ACT C-3') and analyzed by SYBR Green assay in an ABI 7700 Sequence Detector (Applied Biosystems).

**Statistical Analysis**

This was a case-control study. Numerical variables were summarized by mean, median, and range. Nominal variables were summarized according to their distribution of frequencies. Allele and genotype frequencies in CD patients with and without clinical remission were compared by the chi-squared test or, when an expected value was less than 5, by the Fisher exact test; P values of less than 0.05 were considered significant. Odds ratios (ORs) and P values were calculated using the Statistical Package for the Social Sciences (SPSS) version 10.07 for Windows (SPSS Inc., Chicago, Ill). Haplotype frequencies were estimated using the Expectation-Maximisation (EM) algorithm in Arlequin version 2.000 software, with
the number of iterations set at 5000 and initial conditions at 50 and an epsilon value of $10^{-7}$.

**RESULTS**

The cohort of 327 patients with Crohn’s disease consisted of 154 men and 173 women. The median age at diagnosis was 27 years (mean 33, range 8–80) with an interquartile range of 20–37 years. The median duration of follow-up was 10 years (mean 11.57, range 2–47) with an interquartile range of 6–14 years. Characteristics of the 327 patients included in this study are listed in Table 1. One hundred and twelve patients were treated with azathioprine; 36 (32%) were excluded for any of the following reasons: prevention of postoperative recurrence of CD (n = 8), steroid-resistant CD patients (n = 3), duration of drug treatment less than 3 months (n = 25). The most common side effects were nausea and vomiting (7 patients), severe epigastric pain with elevated serum amylase (5 patients). Leucopenia was observed in 6 patients and three patients had abnormal liver enzymes. Other side effects included thrombopenia (n = 1) and flushing (n = 1). Other reasons to early discontinuation of azathioprine were necessity of surgery (n = 1) and no compliance with therapy by a patient (n = 1).

A total of 76 patients (64 steroid-dependent and 12 with perianal fistulizing disease) were included and completed at least three months of azathioprine treatment (Table 1). For these patients remission was achieved in 37 (57.8% of patients) with steroid-dependent CD and in 5 (41.7%) with perianal fistulizing disease. Overall remission rate including all patients was 55.3%. The median dose of azathioprine was 2.3 mg/kg body weight per day (mean 1.96, range 0.5–3). The median duration of follow-up from the start of azathioprine treatment was 2 years (mean 2, range 1–4). No significant differences in dose of azathioprine, follow-up length, phenotypic characteristics, smoking habits or extraintestinal manifestations were found between patients with or without clinical remission (Table 1).

<table>
<thead>
<tr>
<th>Phenotypic characteristics</th>
<th>Crohn’s disease patients N=327</th>
<th>Patients treated with azathioprine N=76</th>
<th>Remission achieved N=42</th>
<th>Remission not achieved N=34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>154 47.1%</td>
<td>36 47.37%</td>
<td>20 47.62%</td>
<td>16 47.06%</td>
</tr>
<tr>
<td>Women</td>
<td>173 52.9%</td>
<td>40 52.63%</td>
<td>22 52.38%</td>
<td>18 52.94%</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1, &lt; 40 years</td>
<td>262 80.1%</td>
<td>60 78.9%</td>
<td>32 76.2%</td>
<td>28 82.4%</td>
</tr>
<tr>
<td>A2, ≥40 years</td>
<td>65 19.9%</td>
<td>16 21.1%</td>
<td>10 23.8%</td>
<td>6 17.7%</td>
</tr>
<tr>
<td>Family history</td>
<td>61 18.7%</td>
<td>17 22.6%</td>
<td>9 21.4%</td>
<td>8 23.5%</td>
</tr>
<tr>
<td>Smokers</td>
<td>143 43.7%</td>
<td>31 40.2%</td>
<td>17 40.5%</td>
<td>14 41.2%</td>
</tr>
<tr>
<td>Disease behaviour</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1, Nonstricturing, nonpenetrating</td>
<td>141 43.1%</td>
<td>30 39.5%</td>
<td>17 40.5%</td>
<td>13 38.2%</td>
</tr>
<tr>
<td>B2, Stricturing</td>
<td>49 15.0%</td>
<td>14 18.4%</td>
<td>8 19.1%</td>
<td>6 17.7%</td>
</tr>
<tr>
<td>B3, Penetrating</td>
<td>137 41.9%</td>
<td>32 42.1%</td>
<td>16 38.1%</td>
<td>16 47.1%</td>
</tr>
<tr>
<td>Location of disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1, terminal ileum</td>
<td>157 48.0%</td>
<td>39 50.9%</td>
<td>21 50.1%</td>
<td>18 52.9%</td>
</tr>
<tr>
<td>L2, colon</td>
<td>54 16.5%</td>
<td>11 14.5%</td>
<td>5 11.9%</td>
<td>6 17.7%</td>
</tr>
<tr>
<td>L3, ileocolon</td>
<td>105 32.1%</td>
<td>23 30.3%</td>
<td>12 28.6%</td>
<td>11 32.4%</td>
</tr>
<tr>
<td>L4, upper gastrointestinal</td>
<td>11 3.4%</td>
<td>3 4.3%</td>
<td>2 4.8%</td>
<td>1 2.9%</td>
</tr>
<tr>
<td>Perianal fistulae</td>
<td>67 20.5%</td>
<td>12 15.78%</td>
<td>5 11.9%</td>
<td>7 20.6%</td>
</tr>
<tr>
<td>Extraintestinal clinical manifestations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutaneous</td>
<td>60 18.3%</td>
<td>16 20.8%</td>
<td>10 23.8%</td>
<td>6 17.7%</td>
</tr>
<tr>
<td>Articular</td>
<td>109 33.3%</td>
<td>29 37.5%</td>
<td>17 40.5%</td>
<td>12 35.3%</td>
</tr>
</tbody>
</table>
In general, 2677TT and 3435TT CD patients were more resistant to azathioprine therapy. The analyses of haplotypes (Table 4) in the Spanish population studied evidenced higher frequency of the double mutant 2677T/3435T haplotype in nonresponders (29.4% versus 20.2%), whereas the wild-type 2677G/3435C haplotype was more abundant in responders (58.3% versus 47.1%). The probability of persistence of Crohn’s symptoms after azathioprine therapy was 1.8-fold in patients with the 2677T/3435T haplotype compared with patients with 2677G/3435C haplotype (OR = 1.8, 95% CI, 0.82–3.9, P = 0.14).

**DISCUSSION**

Our study explores the association with clinical response of two MDR1 single-nucleotide polymorphisms (SNPs; G2677T/A and C3435T) previously suggested to influence PGP expression/function, in a large cohort of white CD patients on azathioprine therapy. Overall, 57.5% of our CD patients on azathioprine therapy successfully achieved disease remission. This rate of remission in CD patients is similar to that reported in previous studies.16,17 Azathioprine is a pro-drug that is converted in vivo to 6-mercaptopurine (6-MP), which is subsequently metabolized to the pharmacologically active 6-thioguanine nucleotides (6-TGN). The latter are also responsible for the cytotoxic side effects associated with this drug. A significant group of IBD patients responds adequately to immunosuppressant therapy with AZA/6-MP, whereas others with seemingly similar patterns do not improve. Azathioprine is characterized by high inter-individual differences in bioavailability and metabolism. No correlation between 6-TGN levels, thiopurine S-methyltransferase (TPMT) activity, AZA/6-MP dose, white blood cells count, and disease activity or remission has been shown previously.21,22 Intestinal absorption of azathioprine ranges from 50% to 72%.23 Reduced drug accumulation is a common mechanism of drug failure. In CD, poor response to

### TABLE 2. Genotype and allele Frequencies of MDR1 G2677T/A Polymorphism in Crohn’s disease patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total numbers</th>
<th>Alleles</th>
<th>G vs T</th>
<th>Genotype</th>
<th>(GG and GT) vs TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>T</td>
<td>A</td>
<td>OR (95% CI) p</td>
</tr>
<tr>
<td>Remission achieved</td>
<td>42</td>
<td>56</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66.67%</td>
<td>33.33%</td>
<td>1.58</td>
<td>71.43%</td>
</tr>
<tr>
<td>Remission not achieved</td>
<td>34</td>
<td>38</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Crohn’s disease patients</td>
<td>327</td>
<td>417</td>
<td>231</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

### TABLE 3. Frequencies of MDR1 C3435T alleles and Genotypes in Patients with Crohn’s disease.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total numbers</th>
<th>Alleles</th>
<th>C vs T</th>
<th>Genotypes</th>
<th>(CC and CT) vs TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>OR (95% CI) p</td>
<td>TT</td>
</tr>
<tr>
<td>Remission achieved</td>
<td>42</td>
<td>60</td>
<td>24</td>
<td>1.55</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71.43%</td>
<td>28.57%</td>
<td>1.55</td>
<td>47.62%</td>
</tr>
<tr>
<td>Remission not achieved</td>
<td>34</td>
<td>42</td>
<td>26</td>
<td>(0.74–3.24)</td>
<td>0.2</td>
</tr>
<tr>
<td>Crohn’s disease patients</td>
<td>310</td>
<td>378</td>
<td>242</td>
<td>39.03%</td>
<td>57</td>
</tr>
</tbody>
</table>
corticosteroids has been associated with high PGP expression.\textsuperscript{12} This study did not investigate the genetic contribution of the \textit{MDR1} gene. PGP is found on the apical surfaces of superficial columnar epithelial cells from the duodenum to the colon.\textsuperscript{8} After oral administration, PGP limits drug absorption by pumping its substrates back into the gut lumen. PGP inhibitors significantly increase intracellular cortisol and cyclosporine levels in human intestinal epithelium and T lymphocytes in a dose-dependent manner, demonstrating a potential mechanism for overcoming poor response to immunosuppressant therapy in refractory inflammatory bowel disease.\textsuperscript{24} Recently, it has been shown that overexpression of PGP yields resistance to 6-mercaptopurine in leucocytes.\textsuperscript{18} Therefore, drug secretion into gut lumen via PGP plays an important role in pharmacokinetics and pharmacodynamics of azathioprine. One might speculate that failure of azathioprine therapy in CD would be related with high expression of PGP in intestinal mucosa and lymphocytes.

The expression and efflux efficiency of PGP is governed, at least in part, by polymorphisms within its encoding gene, \textit{MDR1}. The single change in the nucleotide position C3435T is a synonymous or silent mutation that does not influence the amino acid sequence of the encoded protein, but could alter in the mRNA processing. Controversial studies exist about this polymorphism, some authors suggested a correlation between C3435T and PGP activity,\textsuperscript{25} while others did not show influence of this polymorphism in \textit{MDR1} mRNA expression.\textsuperscript{26} A relevant finding was that this SNP in exon 26 (C3435T) was linked to a G2677T transversion in exon 21 (Ala893Ser).\textsuperscript{27} Indeed, when these two linked SNPs were considered together, a gene-dose effect appeared to be present, suggesting that the Ala893Ser (G2677T) amino acid substitution was associated with lower oral bioavailability of fexofenadine.\textsuperscript{28} These authors observed that 2677TT homozygotes and 3435TT homozygotes had increased net \textit{MDR1} transport activity (as reflected by relatively lower fexofenadine plasma levels). This finding is fully consistent with in vitro expression studies demonstrating enhanced efflux transporting ability of the Ser893 (2677T) variant and the important role of PGP in determining the absorption of its substrates from the gastrointestinal tract after oral administration.\textsuperscript{28}

We have investigated the prevalence of the G2677T/A and C3435T polymorphisms in response to azathioprine in CD patients. In the group of non-responders to azathioprine a higher frequency of the 2677TT genotype (17.65\%)

\[\text{OR} = \frac{1.8}{2.0} = 0.9\text{,} \]

comparing to responders (7.14\%) was observed. Interestingly, in the group of non-responders to azathioprine a higher frequency of individuals with the 3435TT genotype (17.54\%) was found in comparison to clinical-remission achieved CD patients (4.76\%). Our results shows that 2677TT and 3435TT carriers have increased probability to be resistant to azathioprine therapy (2.8 and 4.3 times higher than 2677GG/GT and 3435CC/TC carriers, respectively). Concordantly, recent data suggest that the 3435TT genotype may be useful to predict severe disease (therapy resistance), disease extent and also necessity for surgery in ulcerative colitis.\textsuperscript{3} The G2677T/A and C3435T polymorphisms are in linkage disequilibrium in our population (\(D' = 0.88\); \(P = 4 \times 10^{-40}\))\textsuperscript{29} and therefore we analyzed the distribution of the \textit{MDR1} haplotypes. Our haplotype analyses showed a trend toward a higher frequency of the 2677T/3435T haplotype in non-responders (29.4\%) versus responders (20.2\%) to azathioprine. Moreover, the probability of lack of response to azathioprine therapy was 1.8-fold greater in CD patients with the 2677T/3435T haplotype compared to CD patients with the 2677G/3435C haplotype (OR = 1.8, 95\% CI, 0.82--3.9). Potocnik et al\textsuperscript{4} found 1 haplotype defined by T-T-T alleles in exon 12 (C1236A), in exon 21 (G2677T/A) and in exon 26 (C3435T) significantly associated with higher risk in CD refractory to steroid and azathioprine. In an Italian population of IBD patients, the \textit{MDR1} polymorphisms have no significant role in disease susceptibility and response to medical therapy, but a limitation of this study is the retrospective evaluation of response to medical therapy,\textsuperscript{30} whereas our study prospectively assesses the evaluation of response to azathioprine. Potential reasons for our marginally significant results might include small sample size and difficulty to detect an effect when the con-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Haplotype & Crohn’s disease patients & Remission not achieved & Remission achieved & OR/95\% CI/p 2677T/3435T vs 2677G/3435C to suffer not remission \\
& N(\%) & N(\%) & N(\%) & \\
& n=588 & n=68 & n=84 & \\
\hline
2677G/3435T & 74 (12.45\%) & 10 (14.7\%) & 11 (13.1\%) & \\
2677G/3435C & 296 (50.47\%) & 32 (47.1\%) & 49 (58.33\%) & \\
2677T/3435T & 158 (26.98\%) & 20 (29.4\%) & 17 (20.2\%) & 1.8/0.8 to 3.95/0.14 \\
2677T/3435C & 54 (9.07\%) & 6 (8.8\%) & 7 (8.8\%) & \\
2677A/3435T & 2 (0.3\%) & 0 & 0 & \\
2677A/3435C & 4 (0.6\%) & 0 & 0 & \\
\hline
\end{tabular}
\caption{Estimated two-Loci Haplotypes of C3435T G2677T/A SNPs in Crohn’s disease patients}
\end{table}
tribution of the genetic predisposing factor under investigation is not the only one implicated.

In conclusion, additionally to increase disease susceptibility for CD and ulcerative colitis, these MDR1 polymorphisms may play an important disease-modifying role by modulating the response to immunosuppressant agents. The results of our study indicate a higher frequency of 2677TT/3435T haplotype in azathioprine non-responder CD patients. Diagnostic tests for the discrimination of these MDR1 alleles may provide a potent tool for improving the therapy of CD. Given our suggestive results, additional replications in independent populations with higher statistical power would confirm the real impact of these polymorphisms as genetic markers involved in azathioprine response.

REFERENCES