Accuracy of genotyping using the TaqMan PCR assay for single nucleotide polymorphisms responsible for thiopurine sensitivity in Japanese patients with inflammatory bowel disease

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Abstract. Thiopurine drugs are the most common drugs used to maintain clinical remission in inflammatory bowel disease (IBD). Three single-nucleotide polymorphisms (SNPs), *TPMT* A719G (rs1142345), inosine triphosphate pyrophosphatase (*ITPase*) C94A (rs1127354) and multidrug resistance protein 4 *MRP4* G2269A (rs3765534), have been reported to account for heightened sensitivity to thiopurine drugs in the Japanese population. We investigated the usefulness of the TaqMan® PCR assay (Applied Biosystems) for the rapid detection of these SNPs to improve the safety of thiopurine therapy. We enrolled 44 healthy volunteers and 235 IBD patients. Genotyping of the SNPs was performed using Custom TaqMan SNP genotyping assays, direct sequencing and PCR-RFLP. Genotyping for *MRP4* G2269A by the TaqMan PCR assay was successfully achieved in all samples. Comparison with our previous data using direct sequencing indicated one discordant result, and re-sequencing showed that the TaqMan PCR assay was correct. The overall accuracy of the TaqMan assay for *MRP4* G2269A was 100%. The TaqMan PCR genotyping for *TPMT* A719G and *ITPase* C94A was successfully performed in all samples. The results of *TPMT* A719G by the TaqMan assay were identical with those of PCR-RFLP. In *ITPase* C94A, a comparison of the TaqMan assay and PCR-RFLP yielded 12 discordant results, and direct sequencing showed that the TaqMan PCR assay was correct. The allelic frequency determined by the TaqMan assay was 0.145 for *MRP4* G2269A, 0.009 for *TPMT* A719G and 0.121 for *ITPase* C94A, respectively. In conclusion, the TaqMan® PCR assay is useful for genotyping of SNPs responsible for thiopurine sensitivity in Japanese IBD patients.

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Introduction

The thiopurine drugs, azathioprine (AZA) and 6-mercaptopurine (6-MP), are the most common drugs used to maintain clinical remission in inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC) (1-3). However, in 15-30% of patients, these drugs have to be discontinued due to adverse effects including bone marrow suppression, hepatotoxicity, pancreatitis, fever, rash and gastrointestinal intolerance (4-9). Furthermore, an AZA dosage of 2-3 mg/kg is recommended for the treatment of IBD patients in Western countries (10), but lower doses of AZA (0.6-1.2 mg/kg/day) are used in Japanese patients due to their relatively heightened sensitivity (11).

Considerable interest has focused on the metabolism of thiopurines as a means of individualizing therapy to minimize any adverse effects and to maximize the clinical response. AZA and 6-MP are metabolized to 6-thioguanine nucleotide (6-TGN) (12,13). The cytotoxic and immunosuppressive properties of AZA/6-MP are mediated by 6-TGN, which incorporates into the DNA, thus leading to DNA breakage and an inhibition of immune cell proliferation (12). Susceptibility to bone marrow toxicity upon AZA/6-MP therapy is genetically dependent on inter-individual variations in thiopurine S-methyltransferase (TPMT) enzyme activity, based on the genetic polymorphisms of low-metabolizing alleles (12,13). In the Japanese population, a single nucleotide polymorphism (SNP), *TPMT* A719G, is the most prevalent allele (~2%), and other variants are very rare (7,14-16).

Genetic polymorphism of inosine triphosphate pyrophosphatase (ITPase) has also been suspected as a factor responsible for thiopurine intolerance (5,16-19). ITPase catalyzes the breakdown of inosine triphosphate as part of a futile cycle in the purine metabolic pathway (5). Genetic ITPase deficiency results in the cellular accumulation of thioinosine triphosphate (TITP) and causes thiopurine-associated toxicity (16,19).

Multidrug resistance protein 4 (MRP4/ABCC4) belongs to the MRP family of drug transporters (20-23). MRP4 is localized in the plasma membrane and shows an ATP-dependent transport of a broad range of compounds including nucleoside monophosphate analogs such as 6-MP and 6-TGN. Recently,

Krishnamurthy *et al* identified an SNP in the human *MRP4* gene (G2269A; rs3765534) that dramatically reduces MRP4 function by impairing its cell membrane localization, thus resulting in myelosuppression (21). Database screens suggest a >18.7% allelic frequency of *MRP4* single nucleotide polymorphisms (SNPs) in the Japanese population (21), while it is much less frequent in other populations (21). Direct sequence analyses in our laboratory indicated that the allelic frequency of *MRP4* G2269A was 14.7% in the Japanese population (24). In IBD patients treated with AZA/6-MP, the WBC count was significantly lower in patients with the *MRP4* G2269A SNP than in patients with a wild allelotype (24). Thus, *MRP4* G2269A is a new genetic factor accounting for thiopurine sensitivity in the Japanese population.

SNP genotyping is integrated together with therapy for selecting treatments as well as for monitoring results. Rapid and cost-effective methods must be developed for genotyping, and it would be desirable to include this information in patient records as a guide for physicians to individualize treatment. We previously utilized polymerase-chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses for the determination of the *TPMT* and *ITPase* genotypes (24-26), and used direct-sequencing analysis for *MRP4* (24). However, these methods require multiple steps and are time-consuming.

In this study, we investigated the usefulness of the TaqMan® PCR assay for the rapid detection of SNPs of *TPMT, ITPase* and *MRP4*. This may potentially lead to improved safety and efficacy for thiopurine therapy in individual IBD patients.

Patients and methods

Patients. We enrolled 44 healthy volunteers (female/male: 23/21) and 235 IBD patients (UC, n=157; CD, n=78) attending the Gastroenterology Outpatient Clinic at the Hospital of the Shiga University of Medical Science. All subjects were Japanese. The protocol of this study was approved by the Ethics Committee of the Shiga University of Medical Science.

TaqMan® PCR assay (Applied Biosystems, Foster City, CA). Genotyping for *TPMT* A719G (rs1142345), *ITPase* C94A (rs1127354) and *MRP4* G2269A (rs3765534) was performed using Custom TaqMan SNP genotyping assays in which a fluorogenic probe, consisting of an oligonucleotide labeled with both a fluorescent reporter dye (FAM or VIC) and a quencher dye, is included in a typical PCR. Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence (27,28). Each primer and probe set was used in the TaqMan SNP genotyping assays (ID: C_19567_20, C_27465000_10 and C_27478235_20; Applied Biosystems) in accordance with the information on the Applied Biosystems website (http://www.appliedbiosystems.com).

The PCR was performed according to the manufacturer's instructions provided by Applied Biosystems. The PCR thermal cycling was as follows: initial denaturing at 95˚C for 30 sec; 40 cycles of 92˚C for 5 sec and 60˚C for 20 sec. Thermal cycling was performed using a LightCycler 480 system (Roche Diagnostics, Switzerland). Each 96-well plate contained 80 samples of an unknown genotype and 4 reaction mixtures containing the reagents, but no DNA (no-template control). The no-DNA control samples were necessary for the Sequence Detection System (SDS) 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide. The genotypes were determined visually based on the dye component fluorescent emission data depicted in the X-Y scatter-plot of the SDS software.

Table I. Comparison of the results of the allele distribution determined by the TaqMan assay and direct sequence and PCR-RFLP methods.

	Methods		
Genotype allele	TaqMan	Direct sequence	PCR-RFLP
MRP4 G2289A			
G/G (wild-type)	205	204	
G/A	67	68	
A/A	7	7	
Allele frequency	0.145		
TPMT A719G			
A/A (wild-type)	274		274
G/G	5		5
Allele frequency	0.009		
ITPase C94A			
C/C (wild-type)	215		212
C/A	60		61
A/A	4		6
Allele frequency	0.121		

Total sample number was n=279.

Genotyping of MRP4 G2269A by direct sequencing. Mononuclear cells were isolated from heparinized blood using a Ficoll density gradient. The genomic DNA was isolated using a DNA extraction kit purchased from Qiagen (Valencia, CA). *MRP4* exon 18 encompassing 2269 SNPs was amplified from genomic DNA by the use of forward 5'-TCCAGTGGCTGATTTTCTGA-3' and reverse 5'-GAGTGTAAACTGCGGTGGT-3' primers (21) under the following conditions: 95˚C for 5 min, followed by 32 cycles of 95˚C for 30 sec, 59˚C for 40 sec and 72˚C for 40 sec. The sequencing was carried out on an ABI PRISM 3130 Automated Sequencer (Applied Biosystems, Carlsbad, CA).

Genotyping of TPMT and ITPase. The *TPMT* and *ITPase* genotypes were determined by polymerase-chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses. The PCR primers and PCR protocol were designed according to a previously described method (25,26).

Results

We first genotyped the *MRP4* G2269A (rs3765534) SNP in 279 DNA samples using the TaqMan assay, achieving a success rate of 100% (279/279) (Fig. 1 and Table I). The allelogram showed a clear separation of the *MRP4* G2269A SNPs (Fig. 1). These results were compared with our previous data obtained by the direct-sequencing assay (G/G 204, G/A 68 and A/A 7)

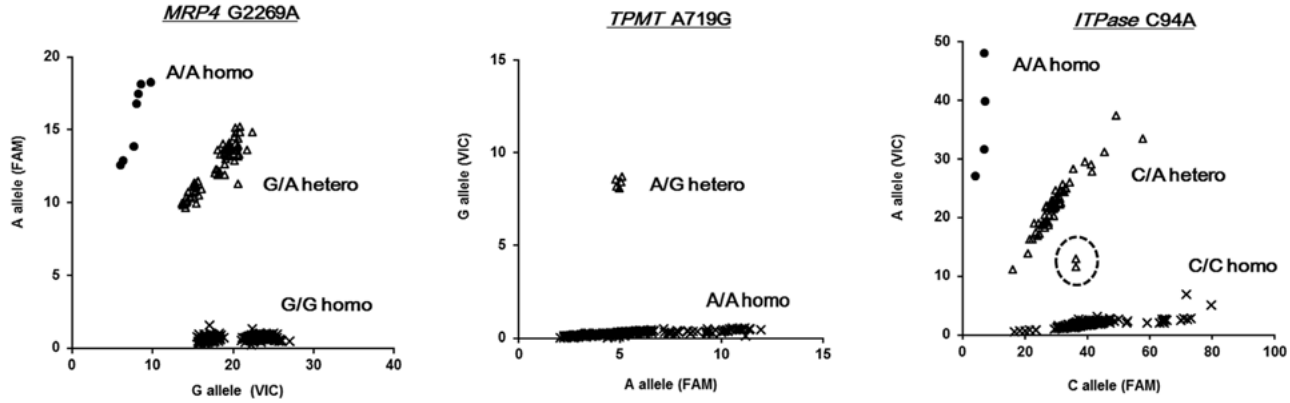

Figure 1. The allelogram obtained by the TaqMan PCR assay. The genotype of two samples in *ITPase* C94A, surrounded by a dotted line, was confirmed by direct-sequencing. These were C/A heterozygotes.

Table II. Allele distribution determined by the TaqMan assay in IBD patients.

Genotype allele	UC	CD	Healthy
MRP4 G2289A			
G/G (wild-type)	117	56	32
G/A	36	21	10
A/A	4	1	2
TPMT A719G			
A/A (wild-type)	155	76	43
G/G	2	2	1
ITPase C94A			
C/C (wild-type)	124	60	31
C/A	30	17	13
A/A	3	1	0

(24) (Table I), which we routinely use for *MRP4* G2269A SNP typing. A comparison of the two methods yielded one discordant result (TaqMan was G/G, and sequencing result was G/A). This was re-sequenced and the TaqMan assay was correct. Thus, we calculated the overall accuracy of the TaqMan assay for *MRP4* G2269A (rs3765534) at 100% (279/279). The allele distribution in IBD patients is shown in Table II. The allele frequency of *MRP4* G2269A was 0.145.

Next, we genotyped *TPMT* A719G (rs1142345) using the TaqMan assay, achieving a success rate of 100% (Fig. 1). The results of *TPMT* A719G were also clearly separated in allelograms, and were identical with the results for PCR-RFLP (Table I). The allele distribution in IBD patients is shown in Table II, and the allele frequency of *TPMT* A719G was 0.009.

The TaqMan assay for *ITPase* C94A also achieved a success rate of 100%. The allelogram showed the two outliers that could not be easily assigned genotypes (dotted circle in *ITPase* C94A allelogram; Fig. 1). These were directly sequenced and assigned to C/A heterozygotes. A comparison between TaqMan assay and PCR-RFLP analyses yielded 12 discordant results for *ITPase* C94A (Table I and III), and direct sequencing indicated that the results of the TaqMan assay were correct. The allele distribution in IBD patients is shown in Table II. The allele frequency of *ITPase* C94A was 0.121.

Table III. Discordant results for *ITPase* C94A determined by PCR-RFLP and TaqMan assay.

PCR-RFLP	TaqMan	n
C/A	C/C	6
C/A	A/A	1
C/C	C/A	3
A/A	C/A	2
Total		12

Direct-sequencing indicates that the results of TaqMan assay are correct.

Discussion

Thiopurines are effective immunosuppressants for IBD patients, but the intracellular accumulation of 6-TGNs causes dose-limiting hematopoietic toxicity. In particular, Japanese populations are sensitive to thiopurines, and relatively lower doses of AZA (0.6-1.2 mg/kg/day) are recommended for the treatment of Japanese IBD patients (11). Similar to *TPMT* A719G and *ITPase* C94A, we recently showed that *MRP4* G2269A is a new factor accounting for heightened thiopurine sensitivity in Japanese patients with IBD (24). Although the SNPs accounting for thiopurine sensitivity have been reported as important factors for individualizing therapy to minimize adverse effects and to maximize clinical response, a rapid and clinically applicable method for the SNP genotyping of these genes has not yet been established.

TaqMan® assays are a reliable method for SNP genotyping (27,29). Good genotyping data can usually be obtained in a short time using a small amount of genomic DNA, and many samples can be processed simultaneously. In the present study, we used the TaqMan assay for the typing of SNPs responsible for thiopurine sensitivity, and accurately genotyped targeted SNPs. In the assays for *ITPase* C94A, we detected two outliers which were confirmed to be C/A heterozygotes by direct sequencing. The precise reasons for this phenomenon remain unclear, but incomplete digestion of the fluorescent-probe by the 5'-nuclease activity of TaqDNA-polymerase was suspected (30). The TaqMan assays for *MRP4* G2269A and *TPMT* A719G

showed no outliers, and clearly separated the genotypes in allelograms.

We routinely use direct sequencing for *MRP4* and PCR-RFLP for *TPMT* and *ITPase* SNP genotyping. There was one discordant result between the TaqMan assays and previously performed sequencing data for *MRP4* G2269A. Re-sequencing indicated that the TaqMan assay was correct. Discordant results for *ITPase* C94A between the TaqMan assays and PCR-RFLPs were detected in 12 samples. Direct-sequencing data supported the accuracy of the results of the TaqMan assays, and incomplete digestion or unclear visualization of the restriction fragments may have caused genotyping errors in the PCR-RFLP assays.

The advantages of the present genotyping method are its accuracy, ease of use and the short processing time. The disadvantage of the present method is the requirement of special PCR machines. The accurate SNP typing of an individual patient among a population with different sensitivities to drugs is critically important for the determination of therapeutic strategies. When 96-well plates are used, the TaqMan assay enables a simultaneous analysis of at least 20 samples for the detection of 3 SNPs. Furthermore, one analysis requires approximately 2 h. Thus, TaqMan assays for SNP genotyping responsible for thiopurine sensitivity may provide a great advantage for the management of IBD patients with thiopurine drug treatment.

In conclusion, we successfully genotyped the SNPs responsible for thiopurine sensitivity using TaqMan® SNP assays. This method was very accurate and convenient. We believe that this technique is useful for the treatment of IBD patients to avoid the adverse events of thiopurine drugs.

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