Effects of Polymorphisms of MDR1, MRPI, and MRP2 Genes on Their mRNA Expression Levels in Duodenal Enteroocytes of Healthy Japanese Subjects

Yuka Moriya, Tsutomu Nakamura, Masanori Horinouchi, Toshiyuki Sakaeida, Takao Tamura, Nobuo Aoyama, Toshiro Shirakawa, Akinobu Gotoh, Sadaki Fujimoto, Masafumi Matsuo, Masato Kasuga, and Katsuhiko Okumura

*Department of Environmental Biochemistry, Kyoto Pharmaceutical University; 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan; b Department of Hospital Pharmacy, School of Medicine, Kobe University; c Department of Endoscopy, School of Medicine, Kobe University; d Departments of Clinical Genetics and International Center for Medical Research, School of Medicine, Kobe University; 7–5–2 Kasumaki-cho, Chuo-ku, Kobe 650–0017, Japan; and e Division of Diabetes, Digestive and Kidney Diseases, Department of Clinical Molecular Medicine; and f Division of Urology, Department of Organs Therapeutics, Faculty of Medicine, Kobe University Graduate School of Medicine; 7–5–2 Kasumaki-cho, Chuo-ku, Kobe 650–0017, Japan. Received May 23, 2002; accepted July 24, 2002

In the present study, we examined whether polymorphisms in the ATP-binding cassette (ABC) transporter genes, MDR1, MRPI and MRP2, were associated with their respective mRNA expression levels in duodenal enterocytes of 13 healthy Japanese volunteers. MDR1 genotypes of T-129C, G2677(A,T) and C3435T, MRPI genotypes of G128C, C218T, G2168A and G3173A, and MRP2 genotypes of C-24T, G1249A, C2302T, C2366T and G4348A were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or direct sequencing. Mutations T-129C, G2677(A,T) and C3435T of MDR1 gene were found at allele frequencies of 2/26, 16/26 and 12/26, respectively. Mutations G2168A of the MRPI gene and C-24T of the MRP2 gene were also found at allele frequencies of 1/26 and 6/26, respectively, whereas other mutations were not detected in MRPI and MRP2 genes. The relative concentrations (mean±S.E.) of MDR1 mRNA to villin mRNA were 0.38±0.15, 0.56±0.14 and 1.13±0.42 in the subjects with C/C3435, C/T3435 and T/T3435, respectively, which supported the lower serum concentrations of digoxin after single oral administration in the subjects with the mutant T-allele at position 3435. Genetic collaboration between positions 3435 and 2677 was suggested, and those in G/G2677, G/(A,T)2677 and T/(A,T)2677 were 0.16±0.05, 1.10±0.40 and 0.63±0.16, respectively (p=0.107). However, there was no remarkable effect of the G2168A of the MRPI gene or of C-24T of the MRP2 gene on the relative MRPI or MRP2 mRNA concentrations, respectively.

Key words P-glycoprotein; multidrug resistance-associated protein; genetic polymorphism; gene expression; duodenal enteroocyte

The small intestine is the primary site of absorption for many drugs administered orally, and P-glycoprotein (MDR1) located in the villus epithelium of the small intestine is considered to play a role in limiting the absorption of xenobiotics.1,2) The extrusive function of the multidrug resistance-associated proteins (MRPs) have also attracted great deal of attention in the small intestine.3) MDR1, MRPI and MRP2 have been reported to show genetic polymorphisms,4—8) and their genotypes are thought to be responsible for the inter-individual differences in absorption properties of the drugs that are their substrates.6,9,10) Kim et al. reported that plasma concentration of fexofenadine after single oral administration was lower in subjects homozygous for the mutant allele at exon 26, position 3435 of the MDR1 gene (T/T3435), than in those homozygous for the wild-type allele (C/C3435).9) We also demonstrated that systemic exposure to digoxin after single oral administration was lower in subjects with the mutation C3435T of the MDR1 gene.10) C3435T is a silent mutation without amino acid substitution, and its effects on phenotype have been discussed from the viewpoint of changes in the level of expression rather than changes in the function of MDR1.6,11) Thus, in the present study, the effects of the MDR1 genotypes of T-129C, G2677(A,T) and C3435T, MRPI genotypes of G128C, C218T, G2168A and G3173A, and MRP2 genotypes of C-24T, G1249A, C2302T, C2366T and G4348A on their mRNA expression levels were examined in human duodenal enterocytes obtained from 13 healthy male Japanese subjects. Since villin is a constitutively expressed and enterocyte-specific protein, the mRNA levels of MDR1, MRPI and MRP2 were expressed relative to the concentration of villin mRNA to estimate expression in the villus tip.12,13)

MATERIALS AND METHODS

Human Duodenal Enterocytes Duodenal enterocytes were obtained as proximal small bowel mucosal biopsy samples from 13 healthy male Japanese volunteers ranging in age from 24—39 years old. The subjects took no medication, and had no significant health problems. After fasting overnight, three or four mucosal biopsy samples were obtained by upper intestinal endoscopy from each subject, and were immediately snap-frozen and stored at −80°C. One biopsy sample obtained from a representative subject, where the expression of mRNAs for target proteins had been confirmed, was used as the authentic standard in each run of the assay. Informed consent was obtained from all subjects prior to their participation in the study. The protocol was approved by the Institutional Review Board of Kobe University Hospital, Kobe University, Japan.

Reverse Transcription (RT) and Real Time Quantitative Polymerase Chain Reaction (PCR) Total RNA was
extracted from the duodenal biopsy samples using an RNase-free Dnase Set (Qiagen, Hilden, Germany) and an RNase-Free Dnase Set (Qiagen) according to the manufacturers’ protocols. Subsequently, poly (A)’ RNA was purified with oligotex-dt30 (Takara Shuzo, Kyoto, Japan). RT and real time quantitative PCR were performed as described previously. The primers and TaqMan probes were designed using the Primer Express 1.0 program (Applied Biosystems, Foster City, CA, U.S.A.). Those for MDR1, MRPI and MRP2 mRNA were described previously. The villin forward primer sequence was 5’-TGACCAGATGCTGATGGTTT-3’ and its reverse primer sequence was 5’-GCCGAGTACTTGGCTCA-3’. The villin TaqMan probe was 5’-TGGTAGAGGACAGAC-3’.

Data Processing and Statistical Analysis  The mRNA levels of MDR1, MRPI and MRP2 were expressed relative to the concentration of villin mRNA. Values are given as the means±standard error. Statistical comparisons were performed by one-way analysis of variance (ANOVA).

MDR1, MRPI and MRP2 Genotyping  Genomic DNA was extracted by the method described previously. In the present study, the mutation T-129C in the promoter region, 1 missense mutation (G2677(A,T)) and 1 silent mutation (C3435T) in the MDR1 gene, 4 missense mutations (G128C, C218T, G2168A and G3173A) in the MRPI gene and 4 missense mutations (G1249A, C2302T, C2366T and G4348A) and the mutation C-24T in the 5’-flanking region in the MRP2 gene were examined. These mutations were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and confirmed by direct sequencing using an automatic ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and the sequencing primers used for PCR amplification. The PCR primers were synthesized by Hokkaido System Science, Co., Ltd. (Sapporo, Japan).

The C3435T genotype of the MDR1 gene was determined as described previously. The following PCR primers were used for G2677(A,T) genotyping: Forward, 5’-TTT GCA GGC TAT AGG TTC CAG-3’; Reverse for G2677A, 5’-GTT TGA CTC ACC TTC CCA G-3’; and Reverse for G2677T, 5’-TTT AGT TTG ACT CAC TTT CCC G-3’. A 226 bp sequence of the MDR1 gene was amplified for G2677A by PCR with the oligonucleotide primers and a TaKaRa Ex Taq (Takara Shuzo Co.), and a 226 bp fragment was amplified for G2677T. PCR consisted of an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min. The temperature was controlled with a programmable heat block (GeneAmp PCR System 9700, Applied Biosystems). After amplification, the PCR product (5 μl) was taken directly from the aqueous phase. DNA was digested with restriction endonucleases in 10 μl of the appropriate basal buffer. To digest the 273 bp PCR product into 135 and 138 bp fragments, 5 units of TseI (New England Biolabal, Inc.) were added to the basal buffer followed by incubation at 65°C for 1 h. PCR products from the T-allele were resistant to TseI digestion. The fragments produced by TseI digestion were separated by agarose gel electrophoresis (3%, 100 V) along with a DNA molecular weight marker (pUC18 Digest, Sigma Chemical Co.) as a reference. The polymorphisms of the MRPI and MRP2 genes were identified in the report of Ito et al.

RESULTS AND DISCUSSION  Among the ABC transporters, MDR1 is the only one in which the effects of genetic polymorphisms on the pharmacokinetics of the substrates have been investigated. Hoffmeyer et al. suggested that mutation C3435T of the MDR1 gene is associated with a lower level of MDR1 expression and a higher plasma concentration of digoxin. On the other hand, plasma concentration of fexofenadine and serum concentration of digoxin were reported to be lower in subjects with the mutant T-129C. In the present study, the relative concentrations (mean±S.E.) of MDR1 mRNA to villin mRNA were 0.38±0.15, 0.56±0.14 and 1.13±0.42 in subjects with C/C3435, C/T3435 and T/T3435, respectively (p=0.154) (Fig. 1). Besides, the C3435T mutation of the MDR1 gene is suggested to be associated with suppression of duodenal absorption rate of digoxin (unpublished data). These results suggested that the higher MDR1 expression in the duodenum was associated with the lower plasma concentration of fexofenadine and serum concentration of digoxin after single oral administration in subjects with the mutant T-allele at position 3435. The silent mutation C3435T has been suggested to be linked with the missense G2677(A,T) producing Ala893Thr and Ala893Ser, respectively. In the present study, the mutations T-129C, G2677(A,T) and C3435T of the MDR1 gene were found at allele frequencies of 2/26, 16/26 and 12/26, respectively, and 3 of 5 subjects with C/C3435 were accompanied with G/G2677, and 3 of 4 subjects with T/T3435 were accompanied with T/T2677, probably due to linkage between positions 3435 and 2677 in the MDR1 gene (Table 1). The relative concentrations of MDR1 trophoresis (5%, 100 V) along with a DNA molecular weight marker (pUC18 Mspl Digest, Sigma Chemical Co., St. Louis, MO, U.S.A.) as a reference. The following PCR primers were used for T-129C genotyping: Forward, 5’-TCA GCA TTC AGT CAA TCC GG-3’; and Reverse, 5’-TCT TCC GGC TGC CCC TAC CTC-3’. These primers were designed using the Primer Express 1.0 program (Applied Biosystems). A 273 bp sequence of the MDR1 gene was amplified by PCR with the oligonucleotide primers and a TaKaRa Ex Taq (Takara Shuzo Co.). PCR consisted of an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, and a final extension step at 72°C for 5 min. The temperature was controlled with a programmable heat block (GeneAmp PCR System 9700, Applied Biosystems). After amplification, the PCR product (5 μl) was taken directly from the aqueous phase. DNA was digested with restriction endonucleases in 10 μl of the appropriate basal buffer. To digest the 273 bp PCR product into 135 and 138 bp fragments, 5 units of TseI (New England Biolabal, Inc.) were added to the basal buffer followed by incubation at 65°C for 1 h. PCR products from the T-allele were resistant to TseI digestion. The fragments produced by TseI digestion were separated by agarose gel electrophoresis (3%, 100 V) along with a DNA molecular weight marker (pUC18 Mspl Digest, Sigma Chemical Co.) as a reference. The polymorphisms of the MRPI and MRP2 genes were identified in the report of Ito et al. 7
mRNA in G/G 2677, G/(A,T) 2677 and T/(A,T) 2677 were 0.16 ± 0.05, 1.10 ± 0.40 and 0.63 ± 0.16, respectively, and the subjects harboring a mutant A- or T-allele tended to have higher level of MDR1 mRNA expression (p = 0.107) (Fig. 1). On the other hand, there was no marked difference in the MDR1 mRNA expression level between subjects with and without the mutant C-allele at position 2129; 0.68 ± 0.19 and 0.57 for T/T 2129 and T/C 2129, respectively (Fig. 1).

The mutations of MRP1 gene, G128C, C218T, G2168A and G3173A, were examined in the present study, but only G2168A was detected. As shown in Table 1, 92% (12/13) of the 13 subjects were homozygous for the wild-type allele (G/G 2168), and only one subject (1/13, 8%) was heterozygous with a mutant A-allele at position 2168 (G/A 2168). In a Japanese population, Ito et al. reported that 4 of 16 mutations, G128C, C218T, G2168A and G3173A, of the MRP1 gene were associated with an amino acid substitution and suggested that G2168A occurred frequently among Japanese subjects.7) There is little information about the level of MRP1 mRNA expression in the small intestine,15,16) and its expression was undetectable in 40% of the biopsy samples examined in the present study. There was no marked difference between the biopsy samples from subjects harboring G/G 2168 and G/A 2168 (0.73 ± 0.21 and 0.57, respectively) (Fig. 2).

Among the mutations C-24T, G1249A, C2302T, C2366T and G4348A in the MRP2 gene, only C-24T was detected in the 13 subjects in the present study, and 61% (8/13) were homozygous for the wild-type allele (C/C 24), 31% (4/13) were compound heterozygotes (C/T 24), and only one subject (1/13, 8%) was homozygous for the mutant allele (T/T 24) (Table 1). There were no remarkable change in the levels of MRP2 mRNA expression; 1.68 ± 0.59, 1.48 ± 0.12 and 2.81, respectively (Fig. 2).

In conclusion, the MDR1 genotypes at position 3435 or 2677 may be useful indexes for MDR1 function, and for the intestinal absorption of MDR1 substrates. In contrast, the mutations in the MRP1 and MRP2 genes were unlikely to be important for its intestinal gene expression.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES


