Table III. Kinetic parameters for *S*-mephenytoin 4′-hydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2C19s.

Variant	K_m[a]	V_{max}		V_{max}/K_m	
		Protein[b]	CYP[c]	Protein[d]	CYP[e]
CYP2C19.1B	33.5 ± 2.5	73.0 ± 25.2	4.64 ± 1.63	2.19 ± 0.77	138 ± 47
CYP2C19.18	42.6 ± 1.6	118 ± 28	3.87 ± 0.92	2.80 ± 0.71	91.2 ± 23.6
CYP2C19.19	99.7 ± 6.6**	62.9 ± 9.3	6.35 ± 1.10	0.63 ± 0.12*	64.3 ± 14.7*

Each value represents the mean ± SD of three separate experiments derived from independent preparations.
[a]μM.
[b]pmol/min/mg protein.
[c]pmol/min/pmol CYP.
[d]μl/min/mg protein.
[e]nl/min/pmol CYP.
*Significantly different from CYP2C19.1B ($p < 0.05$).
**Significantly different from CYP2C19.1B ($p < 0.01$).

have been phenotyped as EMs and PMs (Katsuki et al. 1997; Furuta et al. 1999; Qin et al. 1999). The distribution of EMs and PMs shows wide interethnic differences (Wedlund 2000; Goldstein 2001), and genetic polymorphism in the CYP2C19 gene has been considered responsible for this observation (Goldstein 2001). We recently identified two novel CYP2C19 alleles (*CYP2C19*18* and *CYP2C19*19*) causing amino acid substitutions in a Japanese population (Fukushima-Uesaka et al. 2005), and the functional evaluation of polymorphic CYP2C19 variants should provide important information for individualized drug therapy. In the current study, the enzymatic activities in microsomes from yeast cells expressing wild-type and variant CYP2C19s were investigated by kinetic analysis using *S*-mephenytoin 4′-hydroxylation as a probe for CYP2C19 activity.

The expression of CYP2C19 protein was confirmed by immunoblotting. Furthermore, 450-nm absorbance was measured by reduced CO difference spectra in yeast cell microsomal fractions. The level of CYP2C19 holoproteins of CYP2C19.18 estimated by reduced CO difference spectra was significantly higher than that of CYP2C19.1B, whereas the level of CYP2C19.19 was significantly lower than that of CYP2C19.1B. The profile of functional CYP protein levels among CYP2C19.1B, CYP2C19.18 and CYP2C19.19 was similar to that estimated by immunoblotting. The expression levels of CYP2C19 protein in yeast cells may differ between wild-type and variant CYP2C19s owing to altered stability or folding efficiency of the proteins, as suggested for CYP2D6.10 (Johansson et al. 1994; Fukuda et al. 2000).

S-Mephenytoin 4′-hydroxylation has been suggested to be a representative probe for CYP2C19 (Goldstein et al. 1994; Rendic and Di Carlo 1997). The enzymatic activities in this study were analyzed in two ways: as pmol/min/mg protein (on the basis of microsomal protein level), and as pmol/min/pmol CYP (on the basis of functional CYP protein level). Both variant CYP2C19s were capable of catalyzing *S*-mephenytoin 4′-hydroxylation as well as wild-type CYP2C19 at the substrate concentrations examined. However, the ratio of activity at low and high substrate concentrations on the basis of microsomal and functional CYP protein levels was CYP2C19.19 > CYP2C19.18 ≥ CYP2C19.1B, suggesting that affinity toward *S*-mephenytoin differs among wild-type and variant CYP2C19s.

We further determined the kinetic parameters for *S*-mephenytoin 4′-hydroxylation of wild-type and variant CYP2C19s, and confirmed that the kinetics in all CYP2C19 proteins fits a single enzyme model with typical Michaelis-Menten behavior. The K_m value for

S-mephenytoin 4′-hydroxylation of the wild-type CYP2C19.1B having Ile331Val in this study was comparable to other studies in an expression system using *Escherichia coli* cells (Tsao et al. 2001; Blaisdell et al. 2002). The K_m value of CYP2C19.19 having Ser51Gly/Ile331Val was significantly higher than that of wild-type CYP2C19. Since V_{max} values of CYP2C19.19 on the basis of microsomal and functional CYP protein levels were comparable to wild-type CYP2C19, V_{max}/K_m values were consequently reduced. Therefore, we consider that the reduced affinity of CYP2C19.19 toward *S*-mephenytoin is associated with Ser51Gly substitution. By contrast, the K_m, V_{max} and V_{max}/K_m values of CYP2C19.18 having Arg329His/Ile331Val were similar to those of CYP2C19.1B, suggesting that Arg329His substitution minimally affects the functions of the CYP2C19 enzyme.

The X-ray crystal structures of several mammalian CYP enzymes (rabbit CYP2B4 and CYP2C5, and human CYP2C8, CYP2C9, CYP2D6 and CYP3A4) have been reported to date (Williams et al. 2000, 2003, 2004; Schoch et al. 2004; Scott et al. 2004; Rowland et al. 2006). Lewis (2002, 2003) generated homology modeling for CYP2C19 using the CYP2C5 crystal structure as a template, and identified six substrate recognition sites (SRSs) for CYP2C19. Furthermore, Oda et al. (2004) suggested that Asp293 of CYP2C19 plays an important role in the binding of *S*-mephenytoin, which is surrounded by Val113 and Ala297, and points to the phenyl ring on heme iron. On the other hand, Tsao et al. (2001) suggested that three residues in I helix and SRS-4, Asp286, Ala292 and Leu295 of CYP2C19 are essential for *S*-mephenytoin 4′-hydroxylation using chimeras and mutant enzymes of CYP2C19 and CYP2C9. As stated above, CYP2C19 has been shown to metabolize not only *S*-mephenytoin but also several pharmacologically important therapeutic drugs, and to be involved in interindividual differences in drug metabolism (Katsuki et al. 1997; Furuta et al. 1999; Qin et al. 1999). Interestingly, CYP2C19 and CYP2C9 have very distinctive substrate specificities, although they are the most highly conserved (91% amino acid sequence identity) among human CYP2C enzymes (Romkes et al. 1991; Goldstein and de Morais 1994; Miners et al. 2000; Tsao et al. 2001). For example, His99 of CYP2C19 and Ile99 of CYP2C9 in SRS-1 have been speculated to be important residues for substrate binding (Ibeanu et al. 1996; Niwa et al. 2002). The amino acids of interest in this study, Arg329 and Ser51, are located in the J and A helices, respectively. Although these amino acid residues are not at any SRS or at a binding site for *S*-mephenytoin, the K_m value of CYP2C19 enzyme for *S*-mephenytoin 4′-hydroxylation was increased by Ser51Gly substitution. Further studies are required to identify the metabolic ability of CYP2C19.18 and CYP19.19 toward other substrates.

In conclusion, we expressed two novel variant CYP2C19 enzymes with amino acid substitutions (CYP2C19.18 and CYP2C19.19) found in a Japanese population as well as wild-type CYP2C19 (CYP2C19.1B) in yeast cells, and examined their enzymatic properties using *S*-mephenytoin 4′-hydroxylation. Holo- and apoprotein levels of CYP2C19.18 were significantly higher (1.7–2.0-fold) than those of CYP2C19.1B, and the levels of CYP2C19.19 were significantly reduced to 57–65% of CYP2C19.1B in both spectroscopic and immunoblotting analyses. The K_m value of CYP2C19.19 for *S*-mephenytoin 4′-hydroxylation was significantly higher than that of CYP2C19.1B. Although no significant differences in V_{max} values on the basis of microsomal or functional CYP protein levels were observed between CYP2C19.1B and CYP2C19.19, V_{max}/K_m values of CYP2C19.19 were significantly reduced compared with CYP2C19.1B. By contrast, K_m, V_{max} and V_{max}/K_m values of CYP2C19.18 were similar to those of CYP2C19.1B. These findings suggest that Ser51Gly substitution in CYP2C19.19 decreases affinity toward *S*-mephenytoin of the CYP2C19 enzyme, and may mean that the genetic polymorphism of *CYP2C19*19* also

leads to variations in the clinical response to drugs metabolized by CYP2C19, as reported for *CYP2C19*2* and *CYP2C19*3* (Wedlund 2000; Goldstein 2001).

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SNP Communication

Genetic Variations of the ABC Transporter Gene ABCC3 in a Japanese Population

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: An ATP-binding cassette transporter, multidrug resistance-related protein 3 (MRP3), is encoded by the *ABCC3* gene. The MRP3 protein is expressed in several tissues, and functions as an efflux transporter for conjugated as well as unconjugated substrates. In this study, the 31 *ABCC3* exons and their flanking introns were comprehensively screened for genetic variations in 89 Japanese subjects. Forty-six genetic variations, including 21 novel ones, were found: 8 were located in the 5′-flanking region, 14 in the coding exons (8 synonymous and 6 nonsynonymous variations), and 24 in the introns. Of these 46 variations, five novel nonsynonymous variations, 2221C>T (Gln741Stop), 2395G>A (Val799Met), 2798_2799delAG (Gln933ArgfsX64), 3657C>A (Ser1219Arg), and 4217C>T (Thr1406Met), were found as heterozygous variations. The allele frequencies were 0.011 for Ser1219Arg and 0.006 for the other four variations. Gln741Stop induces a stop codon at codon 741. Gln933ArgfsX64 causes a frame-shift at codon 933, resulting in early termination at codon 997. Both variations result in loss of 6 transmembrane helices (from the 12th to 17th helices) in the C-terminus and all regions of nucleotide binding domain 2. Thus, both variant proteins are assumed to be inactive. These data provide fundamental and useful information for pharmacogenetic studies on MRP3-transported drugs in Japanese.

Key words: *ABCC3*; direct sequencing; novel genetic variation; amino acid change

Introduction

The multidrug resistance-related protein 3 (MRP3) is encoded by the ATP-binding cassette transporter C3 gene (*ABCC3*), and the most homologous to MRP1 among MRP family members.[1] The *ABCC3* gene consists of 31 exons spanning approximately 57 kb at chromosome 17q22, encodes 1527 amino acids, and the resulting membrane proteins (190 and 170 kDa) have different glycosylation patterns.[1-5] MRP3 mRNA and protein are expressed in several tissues including adrenal gland, liver, small intestine, colon, kidney, and pancreas.[1-7] MRP3 protein is localized to the basolateral membranes of the cells in epithelia and thought to play a role in the efflux of organic anions including drugs.[1,5,7]

As of October 22, 2006, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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Similar to MRP1 protein, MRP3 is predicted to have 17 transmembrane helices (TM) in three membrane spanning domains (consisting of TM1 to TM5, TM6 to TM11, and TM12 to TM17) and two large cytoplasmic domains between TM11 and TM12, and downstream of TM17.[1,3] These two cytoplasmic domains contain nucleotide binding domains (NBD1 and NBD2), where binding and hydrolysis of ATP occur to facilitate substrate transport. Three sequence motifs in each NBD are also conserved in the MRP3 protein, called Walker A, Walker B and ABC signature sequence.[1,3]

Human MRP3 can transport glucuronide-, sulfate- and glutathione-conjugates such as the estradiol 17β-D-glucuronide, dinitrophenyl S-glutathione and leukotriene C_4, and also some bile acids including glycocholate.[1,8,9] *In vitro* studies have also indicated that overexpression of MRP3 confers resistance to anticancer drugs etoposide and teniposide.[8,10] In contrast to MRP1 and MRP2, depletion of cellular glutathione did not influence the resistance to etoposide and teniposide, and MRP3 could not efflux glutathione itself.[8,10] The folate analogues methotrexate and leucovorin are also substrates of MRP3.[11] In addition, a recent report showed that an anti-diabetic drug, glyburide, was transported by MRP3.[12] In mice, knock-out of the *ABCC3* gene altered pharmacokinetics of morphine-3-glucuronide, and decreased anti-nociceptive effects of interperitoneally injected morphine-6-glucuronide.[13] Thus, MRP3 is thought to be involved in tissue distribution of anionic compounds, which includes drugs.

MRP3 mRNA and protein expression levels were reported to vary 86- and 84-fold, respectively, in Caucasian livers.[14] These variations may be caused in part by transcriptional regulation. Expression of MRP3 mRNA was enhanced by pregnane X receptor agonists such as rifampicin,[15] and also by omeprazole and β-naphtoflavone, possibly through aryl hydrocarbon receptor pathway.[16] MRP3 expression is also induced by bile salts such as chenodeoxycholic acid through induction of α-1 fetoprotein transcription factor, which binds to the two elements located 224 to 195 bases upstream of the translational start site.[17]

In addition, genetic polymorphisms are known in the *ABCC3* gene. Fifty-one polymorphisms, including 6 nonsynonymous ones, were reported in Caucasians.[14] One polymorphism, $-211C>T$, had a significant correlation with reduced mRNA expression levels in the liver. Saito *et al.* found 35 genetic polymorphisms, including 3 synonymous ones, in 48 Japanese subjects.[18] However, no nonsynonymous polymorphism was reported for Asian populations, which included the Japanese.

In this study, we searched for genetic variations in *ABCC3* by resequencing all 31 exons and their surrounding introns from 89 Japanese subjects.

Materials and Methods

Human genomic DNA samples: Eighty-nine Japanese diabetic subjects administered glimepiride participated in this study with written informed consent. Genomic DNA was extracted from blood leukocytes. The ethical review boards of the International Medical Center of Japan, Nerima General Hospital, and the National Institute of Health Sciences approved this study.

PCR conditions for DNA sequencing and linkage disequilibrium (LD) analysis: Genomic and mRNA sequences of *ABCC3* obtained from GenBank (NT_010783.14 and NM_003786.2, respectively) were used for primer design and as reference sequences. First, multiplex long-range PCR was performed to amplify all 31 exons of *ABCC3* from 100 ng of genomic DNA using 0.025 units/μL of Z-Taq (Takara Bio Inc., Shiga, Japan) with four sets of primers (1 μM) designed in the intronic regions as listed in **Table 1** ("1st PCR"). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon, except for exon 1, was amplified separately in the 2nd PCR using the 1st PCR product as a template by Ex-Taq (0.02 units/μL, Takara Bio Inc.) with primers (0.2 μM) listed in "2nd PCR" in **Table 1**. Because of a high GC content, exon 1 was amplified using 0.05 units/μL of LA-Taq (Takara Bio Inc.) in GC buffer I with 0.5 μM of the primers listed in **Table 1**. The second PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA) and the sequencing primers listed in **Table 1** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany) and the eluates were applied to an ABI Prism 3730 DNA Analyzer (Applied Biosystems). All rare novel variations were confirmed by repeated sequencing analyses of PCR products generated by new genomic DNA amplifications. Under the conditions used, up to 2.1 kb upstream of the translational start site, all exons and their flanking introns were successfully sequenced for all subjects. The nucleotide positions based on the cDNA sequence were numbered from the adenine of the translational initiation site or the nearest exons.

Hardy-Weinberg equilibrium and linkage disequilibrium (LD) analyses were performed with SNPAlyze version 3.1 (Dynacom Co., Yokohama, Japan), and pairwise LDs between variations were obtained for rho square (r^2) and $|D'|$ values.

Table 1. *ABCC3* sequencing primers

	Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	−2k to Exon 1	AGCCTTGGGAAAGCAATAGCGATAG	TCTGTGAGAAGGGAGTTGGACTACA	2,990
	Exons 2 to 17	GCTAAAACCAGAGAAGTCCCATCCA	GAAGCCAGGAGTTCAAGACCAGATG	15,790
	Exons 18 to 26	ACTCCCCCAGTAACCCATTCACGCA	TAAAGACTGAGAGGCAGGAAGGTTG	9,267
	Exons 27 to 31	TTGCCTTTGCCAGTGTGCTCCAGAA	AGACAGAGGAGACCAGAACACTTAG	9,858
2nd PCR	−2k	AACTGTGGGGCTGGAAAGTC	TGTTGGGGCTCAGAAGGAAT	1,015
	−1k	GCAGCCAAGGAAGGAAACAC	AAATACCGAGGCTAACAGTC	1,004
	Exon 1[a]	CCCCCTTGGAAATAGACTCG	GCGCACTCTTCCTCACTTTCC	639
	Exon 2	CACTTGGGACGACAACTGCTC	AGGGCCCAGCCCCATCGGTA	382
	Exons 3 to 4	TGGTGGCTTCAGGTACAAAG	CAAGTGGAGAACAACACAAT	706
	Exons 5 to 6	CTGCCCCAGAGGAGACTGAT	CCTACCCTCATCTGCCCTGT	664
	Exon 7	TTCGGAGGGAGAGCCAGTGA	CTCCAAAGTGCCAGCCATTC	546
	Exon 8	CTTATGCTGTCTGTTCCCCTCCCA	CAAAGGTCACACAGAAAGTGGAGC	481
	Exons 9 to 10	TTGGAGGGGGTGGAGGTTTCAGA	TGCTCCCAGTTCCAGAATAATCC	642
	Exon 11	CCAACCACCATCTTTTGCTT	CCATACTCAGGAAGCAAGGT	335
	Exons 12 to 13	AATGGTGGGCAGCGTGGAAT	TTCCCTGAATCCAATAACTCCCC	685
	Exon 14	AACCTGGACTTTCTCTTTGGGCA	GAAGAGGGAGGAGGTCAGAGTCG	413
	Exons 15 to 17	CTTTCTTCCTCCCTTTTCCCAAG	TGGCACAGAGTTGGGCATAA	945
	Exons 18 to 19	ATCTGCCATCCCAAATAACA	TGTCCAGTTTACTTTACCTC	934
	Exons 20 to 23	CTTTCTTGTTGCCCTTTCAA	GCCCCTAACAAAACTCTGTA	1,492
	Exons 24 to 25	CTGGCTGGGAGGACTCATCT	ACAGTCCTTGAGAACAGACC	709
	Exon 26	AGGTGGTCCTTGGTTAAGTCTGC	ACCTCCTAGCATAGAGCATTGGTTG	488
	Exons 27 to 29	AGGAGTCATTGAACACAAGG	GCTCAATACGAAAGGACAGC	1,559
	Exon 30	AGACCTCTGCTCCGTTCTGG	TCCAGATCTTGGGAAACTCTGC	506
	Exon 31	CAGAACTAACAGAATGGCTA	TTTTGGCTGTGAAGGCAATA	950
Sequencing[b]	−2k	AACTGTGGGGCTGGAAAGTC	GCTTTACACCTTGTTACGGC	
		TTAGCAGGGAAGACAGACTC	TGTTGGGGCTCAGAAGGAAT	
	−1k	GCAGCCAAGGAAGGAAACAC	ATTGAATGGAAAGGGTAGGC	
		AAGTGTGCCAGGAAATACGC	AAATACCGAGGCTAACAGTC	
	Exon 2	CACTTGGGACGACAACTGCTC	GAGAATCGCTTGAACCCAGA	
			AGGGCCCAGCCCCATCGGTA	
	Exon 15	CTTTCTTCCTCCCTTTTCCCAAG	AACCAAGCCCTCCGCCCTTCCAGA	
	Exons 16 to 17	TCTGGAAGGGCGGAGGGCTTGGTT	TGGCACAGAGTTGGGCATAA	
	Exon 18	ATCTGCCATCCCAAATAACA	AGTTGTCCCCACAGGTTTCT	
	Exon 19	ATGGGCACACTTCACACTCA	TGTCCAGTTTACTTTACCTC	
	Exons 20 to 21	CTTTCTTGTTGCCCTTTCAA	AATCCCAGAACACACTGAGC	
	Exon 22	ACTGTGAGTCGGTGGGGCAA	GAATCAGAGACACGGGTTGA	
	Exon 23	AACCCGTGTCTCTGATTCTG	GCCCCTAACAAAACTCTGTA	
	Exon 24	CTGGCTGGGAGGACTCATCT	CAGATGAGGAGGAAATGGGG	
	Exon 25	TGTCCCTCCTTTCCCCTAAG	ACAGTCCTTGAGAACAGACC	
	Exon 26	TCATTCGTGATTACAGCCCC	ATTGGTTGATGCTCAGTAAG	
	Exon 27	AGGAGTCATTGAACACAAGG	GAGAAGGGAAGGGGCTCTGA	
	Exon 28	GTCTTTTGAGAGCACAAGTG	ATGACGGGTGGCTCACTGAT	
	Exon 29	CTGGAAACAGAGTGGGAATG	GCTCAATACGAAAGGACAGC	
	Exon 31	CAGAACTAACAGAATGGCTA	AAAGCATTCAGCACAGTTAC	
		CAAACACTGGGGGCACCTTA	TTTTGGCTGTGAAGGCAATA	

[a]LA-Taq with GC buffer I was used for amplification due to a high GC content in this region.
[b]For exons 1, 3 to 14, and 30, the same primers were used for both the 2nd PCR and sequencing.

Results and Discussion

We found 46 genetic variations, including 21 novel ones, from 89 Japanese subjects (**Table 2**). Of these variations, 8 were located in the 5′-flanking region, 14 in the coding exons (8 synonymous and 6 nonsynonymous variations), and 24 in the introns. All detected variations were in Hardy-Weinberg equilibrium ($p>0.05$) except for 3 variations, −1134C>T, IVS7+172C>G and IVS25−25C>T. These deviations were probably caused by the small number of subjects analyzed.

Five novel nonsynonymous variations, 2221C>T (Gln741Stop), 2395G>A (Val799Met), 2798 2799-delAG (Gln933ArgfsX64), 3657C>A (Ser1219Arg), and 4217C>T (Thr1406Met), were found as heterozygous variations. The allele frequencies were 0.011 for Ser1219Arg, and 0.006 for the other four variations. Gln741Stop induces the early stop codon at codon 741,

Table 2. Summary of *ABCC3* variations detected in this study

SNP ID					Position				
This Study	dbSNP (NCBI)	Pharm GKB[b]	Reference	Location	NT_010783.14	From the translational initiation site or from the end of the nearest exon[c]	Nucleotide change	Amino acid change	Frequency
MPJ6_AC3001	rs1989983		14	5′-flanking	7363809	−1767	GAAGTCCCAGAGG>ACATCAAGGAGCT		0.169
MPJ6_AC3002[a]				5′-flanking	7363965	−1611	CCGTAACAAGGTG>ATAAAGCTCTGTA		0.011
MPJ6_AC3003			14	5′-flanking	7364363	−1213	GGTGAAACTGGAC>GAGACCTGTGGGC		0.118
MPJ6_AC3004	rs4148403		18	5′-flanking	7364442	−1134	AGCCCCAACAAGC>TGGTGCTGAGTTG		0.101
MPJ6_AC3005	rs4148404		18	5′-flanking	7364679	−897	TCACCTGTCCTT/del C/CCCCCCCAACCC		0.118
MPJ6_AC3006[a]				5′-flanking	7364711	−865	GCTGAAGCAGAGG>AGAATTCACACAT		0.006
MPJ6_AC3007	rs9895420		14	5′-flanking	7365316	−260	CATCCCCCTGGCT>ATGGCCCAGGGGC		0.118
MPJ6_AC3008	rs4793665		14	5′-flanking	7365365	−211	CAAGGGCCCCCCC>TACCTCTGCCCCA		0.837
MPJ6_AC3009	rs11568609	#		Exon 1	7365607	32	CCGGGGAGCTCGG>ACTCCAAGTTCTG	Gly11Asp	0.006
MPJ6_AC3010[a]				Intron 1	7365799	IVS1+179	GCGCCGCCCGGAG>ACCGGGTCCCACG		0.045
MPJ6_AC3011[a]				Exon 2	7386560	135	CTGCATCTACCTG>TTGGGTCGCCCTG	Leu45Leu	0.028
MPJ6_AC3012	rs2301836	#	18	Intron 3	7387632	IVS3−53	GGGAGAAATGGAG>AGCAGGTCCAGAT		0.174
MPJ6_AC3013	rs739923	#	18	Intron 5	7389052	IVS5−22	CCTGATTCCCCCG>ATCCTATTCTCTC		0.253
MPJ6_AC3014[a]				Intron 7	7390179	IVS7+172	AGGCCTGGATGGC>GGCTAGTTCTCCC		0.331
MPJ6_AC3015	rs2301837	#	18	Intron 7	7391544	IVS7−18	TAACCCACTGCTC>TCTTCCTCCCTGG		0.146
MPJ6_AC3016[a]				Exon 10	7394669	1257	GGATGCCCAGCGC>TTTCATGGACCTT	Arg419Arg	0.006
MPJ6_AC3017	rs4148414		18	Intron 12	7398481	IVS12−21	ACTCTACCCTGA/del C/ACCACCTCCACG		0.028
MPJ6_AC3018	rs16949205			Intron 14	7399266	IVS14+110	TCTTCCTCTGTTC>GACATCTGCTTCG		0.034
MPJ6_AC3019	rs879459		18	Intron 14	7399413	IVS14−79	CTGTCCTCCTTTC>TCCCTGCCCCCCA		0.815
MPJ6_AC3020[a]				Exon 17	7400114	2188	GCCTGTGCCTTGC>TTAGCTGACCTGG	Leu730Leu	0.006
MPJ6_AC3021[a]				Exon 17	7400147	2221	CCTGGTGGGGATC>TAGACAGAGATTG	Gln741Stop	0.006
MPJ6_AC3022	rs4794176	#	14	Intron 17	7400201	IVS17+34	CTAAGAGGCTAGG>CGCATAGAGCTGC		0.815
MPJ6_AC3023[a]				Intron 17	7403602	IVS17−8	CACCCCCCGCCTC>TCCTCCAGGGCAT		0.006
MPJ6_AC3024[a]				Exon 18	7403756	2388	CGTCATCGGGCCA>GGAAGGCGTGCTG	Pro796Pro	0.011
MPJ6_AC3025[a]				Exon 18	7403763	2395	GGGCCAGAAGGCG>ATGCTGGCAGGCA	Val799Met	0.006
MPJ6_AC3026[a]				Intron 18	7403874	IVS18+97	AGACTTGGAGGTG>ATGGGGGGCGCAA		0.022
MPJ6_AC3027	rs2240801	#	18	Intron 18	7404080	IVS18−28	GGAGGGTGGTAGG>AGGTGAGAGCCTG		0.017
MPJ6_AC3028[a]				Intron 19	7404392	IVS19+95	CCTAGTGTTGTGC>TCAGGCAGGTTCT		0.449
MPJ6_AC3029	rs4148415	#	18	Intron 19	7405878	IVS19−123	GCCCTTTCAATCC>TCCCTCATTTTAT		0.478
MPJ6_AC3030[a]				Intron 19	7405928	IVS19−73	TTCATTAGAGTGG>CGGAATGGGAGAT		0.006
MPJ6_AC3031[a]				Exon 20	7406077	2676	TCTGACAGACAAT>CGATCCAGTCACC	Asn892Asn	0.006
MPJ6_AC3032	rs2072365	#	18	Intron 20	7406144	IVS20+29	TCCCAGCCCTCCC>TGGAGGCTGTATC		0.478
MPJ6_AC3033	rs2072366	#	18	Intron 20	7406168	IVS20+53	CAGGCCTCCCCAA>GGCCCTGCCAGAT		0.169
MPJ6_AC3034[a]				Exon 21	7406353_7406354	2798_2799	AGAGAAGGTGC/del AG/GTGACAGAGGC	Gln933ArgfsX64	0.006
MPJ6_AC3035	rs4148416	#	18	Exon 22	7406701	3039	CCTGAGGCTGGGC>TGTCTATGCTGCT	Gly1013Gly	0.096
MPJ6_AC3036	rs8077268	#	14	Intron 22	7406800	IVS22+71	GGCCCCCCAAACC>TGTGCCCTTGCAT		0.017
MPJ6_AC3037[a]				Intron 23	7407236	IVS23+9	GCAGGTGTGGGGT>AGGGCGTGATTCC		0.006
MPJ6_AC3038	rs967935	#	18	Intron 24	7408666	IVS24−66	TGTCCCTCCTTTC>TCCCTAAGCAGAA		0.096
MPJ6_AC3039	rs11568583	#		Intron 24	7408728	IVS24−4	ATCTGATCCCCCA>GTAGGTGGCTGAG		0.039
MPJ6_AC3040[a]				Exon 25	7408810	3657	CATCGGGAGGAGC>AAGCCTGAACCCG	Ser1219Arg	0.011
MPJ6_AC3041[a]				Intron 25	7410412	IVS25−25	GAGATCGCCATAC>TGTATAACCCAGT		0.011
MPJ6_AC3042[a]				Intron 26	7410544	IVS26+6	GACAGAGGTGGGT>AACTGGCATGAGC		0.006
MPJ6_AC3043	rs2277624	#	18	Exon 27	7414383	3942	TCTGCATGTGCAC>TGGTGGCGAGAAG	His1314His	0.174
MPJ6_AC3044	rs872793		18	Intron 28	7415158	IVS28−190	AGGTCTTCGTGAT>CTGGCAAAGCTGA		0.101
MPJ6_AC3045[a]				Exon 29	7415451	4217	CCCACCTGCACAC>TGTTTGTGAGCTC	Thr1406Met	0.006
MPJ6_AC3046	rs1051640	#	18	Exon 31	7421764	4509	AGTAGTAGCTGAA>GTTTGATTCTCCA	Glu1503Glu	0.039

[a]Novel variations detected in this study.

[b]Variations included in the PharmGKB database are indicated by "#".

[c]The exon-intron boundary and base numbering are based on Genbank accession NM_003786.2.

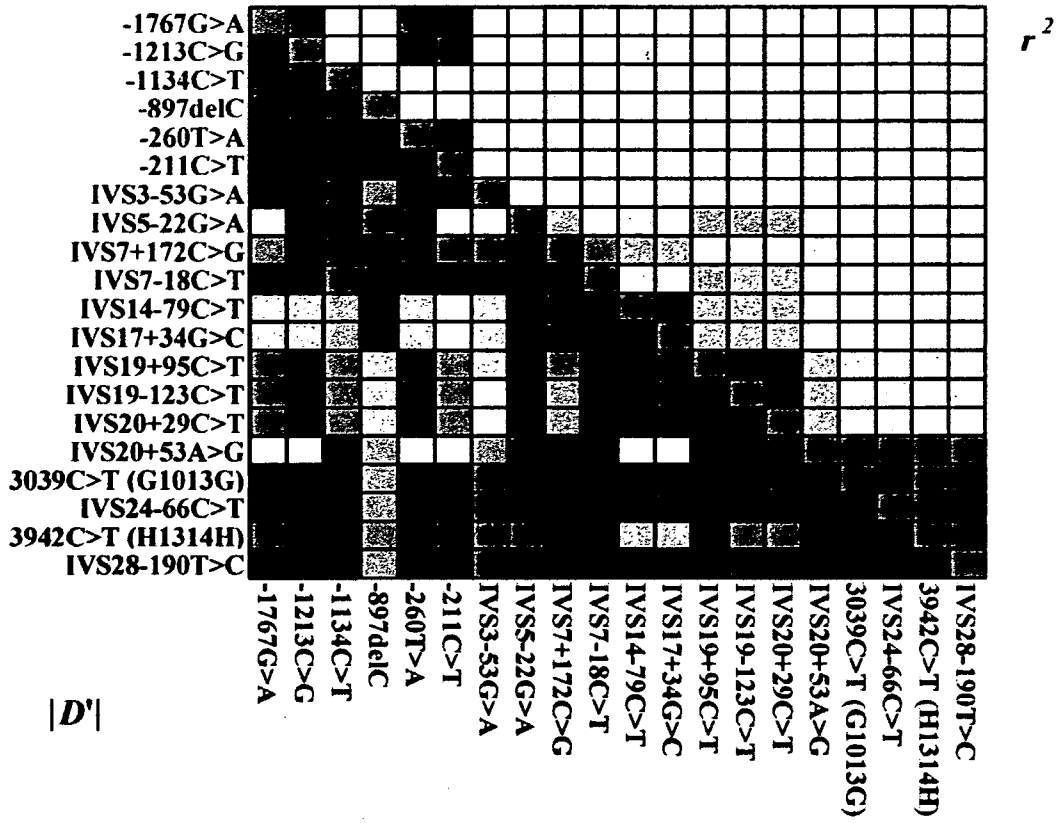

Fig. 1. Linkage disequilibrium (LD) analysis of *ABCC3*. Pairwise LD is expressed as r^2 (upper right) and $|D'|$ (lower left) values (from 0 to 1) by 10-graded blue colors. A denser color represents closer linkage.

resulting in loss of the signature sequence and the Walker B motif of NBD1, TM12 to TM17, and all regions of NBD2. Variation Gln933ArgfsX64 causes a frame-shift from codon 933, and termination at codon 997, which also results in loss of TM12 to TM17, and all regions of NBD2. Thus, both variations would generate truncated and inactive proteins. The other three variations, Val799Met, Ser1219Arg, and Thr1406Met, were located 24 bases downstream of the Walker B motif in NBD1, in the extracellular loop between TM16 and TM17, and 20 bases upstream of the signature sequence in NBD2, respectively. In MRP1, the corresponding amino acid residues for Val799, Ser1219, and Thr1406 are methionine, histidine, and aspartic acid, respectively. By the SIFT program (http://blocks.fhcrc.org/sift/SIFT.html), which predicts the functional effects of amino acid substitutions based on sequence homology and physical properties of amino acids,[19] Thr1406Met is expected to affect protein function. The functional significance of these three substitutions should be clarified in future studies.

We also detected one known nonsynonymous variation 32G>A (Gly11Asp) at a 0.006 frequency. We did not detect previously reported variations with ≥ 0.01 frequencies in Caucasians: 202C>T (His68Tyr, at 0.016), and 3890G>A (Arg1297His; at 0.052).[14] Thus, it is likely that these SNPs are ethnic-specific.

Another known polymorphism in the 5′-flanking region, −211C>T, was reported to be significantly associated with reduced hepatic mRNA expression.[14] The allele frequency of −211C>T in Japanese was 0.837, which is 66% higher than that in Caucasians (frequency: 0.505). Thus, it is assumed that the subjects with low mRNA levels are dominant in Japanese.

Using the 20 genetic variations detected at ≥ 0.05 frequencies, LD analysis was performed with the r^2 and $|D'|$ statistics, and the pairwise values for both are shown with 10-graded blue colors in **Fig. 1**.

For the r^2 values, perfect LD ($r^2 = 1$) was detected between −1213C>G and −260T>A, and very strong LD ($r^2 = 0.96$) was observed between −1767G>A and −211C>T. These two combinations were moderately linked with each other ($r^2 \geq 0.65$). IVS14−79C>T and IVS17+34G>C were also in complete LD ($r^2 = 1$). Strong LDs were also observed among IVS19+95C>T, IVS19−123C>T and IVS20+29C>T ($r^2 \geq 0.89$). Moderate to strong links were detected among the five variations, IVS20+53A>G, 3039C>T (Gly1013Gly),

IVS24−66C>T, 3942C>T (His1314His), and IVS28−190T>C ($1 \geq r^2 \geq 0.50$). The other pairs had r^2 values below 0.5.

For the $|D'|$ values, strong linkages ($|D'| \geq 0.90$) were observed in all pairs among 6 variations from −1767G>A to −211C>T, among 3 variations from IVS5−22G>A to IVS7−18C>T, among 6 variations from IVS7−18C>T to IVS20+29C>T, among 6 variations from IVS19+95C>T to IVS24−66C>T, and among 4 variations from 3039C>T (Gly1013Gly) to IVS28−190T>C. However, due to relatively sparse variations in this large gene and the complicated LD patterns, clear LD blocks could not be defined.

In conclusion, we identified 46 genetic variations, including 21 novel ones, in 89 Japanese subjects in *ABCC3*. Two novel variations result in generation of truncated proteins, and three in amino acid substitutions. This information would be useful for pharmacogenetic studies to investigate the associations of *ABCC3* variations with interindividual differences in drug disposition.

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SNP Communication

Genetic Variations and Haplotype Structures of Transcriptional Factor Nrf2 and Its Cytosolic Reservoir Protein Keap1 in Japanese

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Transcriptional factor Nrf2 and its cytosolic reservoir protein Keap1 play important roles in induction of the expression of genes for xenobiotic metabolism and disposition, many of which are involved in protection from oxidative stress. In this study, 5 *NFE2L2* (encoding Nrf2) and 6 *KEAP1* exons and their flanking introns were comprehensively screened for genetic variations in 84 Japanese subjects. As for *NFE2L2*, 14 genetic variations were found, including 9 novel ones: 7 were located in the 5′-flanking region, 1 in the 5′-untranslated region (5′-UTR), 3 (1 synonymous and 2 nonsynonymous) in the coding exons, 1 in the intron, and 2 in the 3′-UTR. Two novel nonsynonymous variations, 697C>T (Pro233Ser) and 1094G>T (Ser365Ile), were heterozygously found with allele frequencies of 0.012 and 0.006, respectively. Regarding *KEAP1*, 18 genetic variations were detected, including 13 novel ones: 2 were located in the 5′-flanking region, 4 in the coding exons (4 synonymous), 5 in the introns, 4 in the 3′-UTR, and 3 in the 3′-flanking region. Based on the linkage disequilibrium (LD) profiles, both genes were analyzed as single LD blocks, where 14 (*NFE2L2*) and 18 (*KEAP1*) haplotypes were inferred. Six (*NFE2L2*) and 5 (*KEAP1*) haplotypes were relatively prevalent ($\geq$0.03 frequencies) and accounted for $\geq$88% of the inferred haplotypes. Haplotype-tagging variations of each gene were identified to capture these prevalent haplotypes. These data would be fundamental and useful information for pharmacogenetic studies on Nrf2-regulated genes for xenobiotic metabolism and disposition.

Key words: ***NFE2L2*; *KEAP1*; novel genetic variation; amino acid change; haplotype**

Introduction

Electrophiles and radicals are sometimes generated as intermediates or by-products in the metabolism of xenobiotics including drugs.[1,2] These molecules provoke oxidation of lipids and DNA, and generate protein- and DNA-adducts, which contribute to various tissue injuries, carcinogenesis, and autoimmune diseases. Several enzymes such as glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs)

As of January 9, 2007, the novel variations reported here are not found in the database of Japanese Single Nucleotide Polymorphisms (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB Database (http://www.pharmgkb.org/).

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can detoxify these harmful molecules.[3,4] Conjugated molecules are exported from cells *via* multidrug resistance-associated proteins (MRPs) such as MRP1 and MRP2.[5] Thus, expression of these enzymes and transporters is important for cellular defense against oxidative stress.

Recently, Nrf2, which belongs to the basic leucine zipper transcriptional factor family, has been shown to play a key role in inducing the diverse defensive genes against oxidative stress.[3,4,6] Nrf2 knockout mice demonstrated a greater severity in hepatic damage and a higher mortality toward a high dose of acetaminophen than normal mice.[7] Nrf2 mRNA is ubiquitously expressed in human tissues and is highly expressed in the kidney and lung.[8] Under normal (non-stress) conditions, Nrf2 is present with its reservoir protein Keap1 in the cytosol and is constitutively degraded by the ubiquitin-proteasome pathway. Under oxidative stress conditions, Nrf2 is released from Keap1, is translocated into the nucleus, undergoes heterodimerization with small Maf proteins, and binds to antioxidant-responsive elements of the target genes, and finally induces expression of these genes. In this Nrf2-Keap1 system, Keap1 is thought to act as a redox sensor through 3 cysteines at residues 151, 273, and 288.[3]

In humans, Nrf2 ligands have been reported to induce several drug metabolizing enzymes (including UGT1A1, GSTA1, and GSTA2), transporters (such as ABCC2/MRP2 and SLC22A6/OAT1), and many anti-oxidative enzymes (such as heme oxygenase-1 and NAD[P]H: quinone oxidoreductase 1).[6,9-11] It is possible that genetic variations of *NFE2L2* encoding Nrf2 protein and *KEAP1* affect the expression of these enzymes and transporters, and thus influence the susceptibility to electrophiles generated from drugs and other xenobiotics. As for *NFE2L2*, single nucleotide polymorphisms in the 5′-flanking region (3 polymorphisms) and 5′-untranslated region (5′-UTR) (1 polymorphism) were previously found in 12 Japanese subjects.[12] However, nonsynonymous variations have not been found in the Japanese. In order to search genetic variations of the candidate genes that may be associated with secondary failure to anti-diabetic drugs sulfonylureas, the 5′-regulatory region, all exons and their surrounding introns of *NFE2L2* and *KEAP1* were sequenced in 84 Japanese subjects. Linkage disequilibrium (LD) and haplotype analyses were also performed using the detected variations of both genes.

Materials and Methods

Human genomic DNA samples: Eighty-four Japanese type 2 diabetic subjects, who were administered anti-diabetic drugs (sulfonylureas), participated in this study. The patients consisted of 60 males and 24 females, and were at ages of 66.8±11.3 years old (average±SD). Their period of diabetes was 17.9±10.1 years. The ethical review boards of the International Medical Center of Japan and the National Institute of Health Sciences approved the pharmacogenetic study on secondary failure to sulfonylureas. Written informed consent was obtained from all subjects. Genomic DNA was extracted from the blood leukocytes and then amplified by GenomiPhi DNA Amplification Kit (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA).

PCR conditions for DNA sequencing: The following sequences obtained from GenBank were used for primer design and as the reference sequences: NT_005403.16 (genome) and NM_006164.2 (mRNA) for *NFE2L2* and NT_011295.10 (genome) and NM_203500.1 (mRNA) for *KEAP1*. For sequencing, multiplex long-range PCR was performed to amplify all 5 exons of *NFE2L2* or all 6 exons of *KEAP1* from 50 ng of genomic DNA using 0.025 units/μL of Z-Taq (Takara Bio Inc., Shiga, Japan) with two sets of primers (1 μM) designed in the intronic regions as listed in "1st PCR" of **Table 1** (*NFE2L2*) or **Table 2** (*KEAP1*). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon was separately amplified in the 2nd PCR using the 1st PCR product as a template by Ex-Taq (0.02 units/μL, Takara Bio Inc.) with the primers (0.2 μM) listed in "2nd PCR" in **Table 1** (*NFE2L2*) or **Table 2** (*KEAP1*), except for the following regions: −0.5 kb promoter region and exon 1 for *NFE2L2*, and exon 1 (variants 1 and 2) to exon 3 for *KEAP1*. Because of a high GC content, these regions were amplified using 0.05 units/μL of LA-Taq (Takara Bio Inc.) in GC buffer I with 0.5 μM of the primers listed in **Tables 1 and 2**. The second PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and the sequencing primers listed in **Tables 1 and 2** (Sequencing). Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany) and the eluates were applied to an ABI Prism 3730 DNA Analyzer (Applied Biosystems). All relatively low frequent variations (including novel nonsynonymous ones of *NFE2L2*, and variations of *KEAP1* deviated from Hardy-Weinberg equilibrium) were confirmed by repeated sequencing analyses of PCR products generated from original (not amplified) genomic DNA. Under the conditions used, up to 1.1 kb (*NFE2L2*) and 130 bases (*KEAP1*) upstream of the transcriptional start site, all exons (including non-coding exon 1s and the region between the exon 1s of *KEAP1*) and their flanking introns were successfully

Table 1. Primers used for sequencing *NFE2L2*

	Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	−1 k to Exon 1	GGCTCATTGTCTACCTTCTCTGATG	GCTCTCCTTACTGCCCTGTTTATCA	4,052
	Exon 2 to Exon 5	TGGGTAGAAACAGGTGTCACTATGG	GTGCTAAAGTGAGTTGCTAAACGCC	7,140
2nd PCR	−1 k	CTATCCTCTTTGAACCCTTA	CTCGTGTTCGCAGTCACCCT	565
	−0.5 k[a]	CTTAGGAGAATGGAGACACG	CACAAGGGCGGGGCAAGAGT	609
	Exon 1[a]	GGTGGGGGATTTTCGGAAGC	CCCAGACCTTCCCGCAACTT	567
	Exon 2	GATGGCTATGTGTTTGTAGG	GTTAGGTACTGAACTCATCA	652
	Exon 3	GTGTGTGGCAAGCACTGTTT	GCAATAGTCAATGGTTTTGG	663
	Exon 4	TGAGTCAGTGGGGTAGGAAA	TCTATCCTCAAGATGTCCAA	534
	Exon 5	CTGAAGATAATGTGGGTAGG	ACTGGGACTTGTGTTTAGTG	560
		AACCAAAACCACCCTGAAAG	AGGTGCTGAGTTGTTTTTTC	694
		ACAAAAGCCTTCACCTACTG	GCGTATGTCTACTGATGGAA	927
Sequencing[b]	−0.5 k	GGTCCAAATCTTTAGCCCCC	CACAAGGGCGGGGCAAGAGT	
		CTTAGGAGAATGGAGACACG		
	Exon 3	GTGTGTGGCAAGCACTGTTT	ATTTATAGGCTAAGGTTTCC	
	Exon 5	CTGAAGATAATGTGGGTAGG	ACTGGGACTTGTGTTTAGTG	
		AACCAAAACCACCCTGAAAG	AGGTGCTGAGTTGTTTTTTC	
		ACAAAAGCCTTCACCTACTG	CATACTGACACTCCAATGCT	
		AGCATTGGAGTGTCAGTATG	GCGTATGTCTACTGATGGAA	

[a] LA-Taq with GC buffer I was used for amplification.
[b] For sequencing the −1 k region and exons 1, 2 and 4, primers for the 2nd PCR were also used.

Table 2. Primers used for sequencing *KEAP1*

	Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Exon 1 to Exon 2	CTGAACCATAGAGAACTAAGTAAGC	GTCAGGCACTCGTCTATGTAATCAG	7,304
	Exon 3 to Exon 6	GACTCTGTCTCAAAAACCAAAGCCA	TTAGAGTCAGGGGTATTGCTGTGTC	8,818
2nd PCR	Exon 1 (variant 1)[a]	GAGACATCCAGCAACGAAAT	TGTCACAATAAAAGTCCCCG	708
	Exon 1 (variant 2)[a]	TTGGGCAGCAGCGACGGGGA	CTGGTTCTCTCGCTCGGTTT	611
	Exon 2[a]	CAAATGGATTCTGCTTCACC	CCCCAGTTGTTTTTTGTTTGTGTG	1,079
	Exon 3[a]	ATCCCCATTTAGCAGATAAG	AGTCCCCTAAGCATTTCCCA	1,016
	Exon 4	CCCAGGTTCAAGTGATTATCTCGC	GACTCTATCAGAATCCAGGG	673
	Exon 5	AGCCCTGGATTCTGATAGAG	ACACCATCTCAAAAGAAAAAAAAC	608
	Exon 6	GTAAGAGACTAAGGTTTTGC	AAAAAATGACCTTGGGGAGC	1,157
Sequencing[b]	Exon 2	ATTCTGCTTCACCTACTTTG	CCGCAGCCCGTTGGTGAACA	
		CTCATCCAGCCCTGTCTTCA	GTGTGTTTGTTTGTTTAGAG	
	Exon 3	CATTTAGCAGATAAGGAAGC	TCACCTGCGTGGGCTTGTGC	
		GCACAAGCCCACGCAGGTGA	CCCCTAAGCATTTCCCAGCC	
	Exon 4	GAAAAAAAAGAGTATCTGGC	GCAAAAGCAAAAGCAGTCCACAAA	
	Exon 6	GAGACTAAGGTTTTGCTATG	CTCTTTCCACACCCCCTTTC	
		GACATCTCAAAAGAAGTCCA	GCTGAAACTGAAGGACAACT	

[a] LA-Taq with GC buffer I was used for amplification.
[b] For sequencing exons 1 and 5, primers for the 2nd PCR were also used.

sequenced for all subjects. The nucleotide positions based on the cDNA sequence were numbered from the adenine of the translational initiation site or the nearest exon.

Linkage disequilibrium (LD) and haplotype analyses: Hardy-Weinberg equilibrium and LD analyses were performed by SNPAlyze software ver. 3.1 (Dynacom Co., Yokohama, Japan), and pairwise LDs between variations were obtained for the frequently used coefficients, $|D'|$ and rho square (r^2).[13] $|D'|$ was used to assess the probability for past recombinations, and r^2 was used as a parameter for the linkage between a pair of variations. Some of the haplotypes were unambiguous from subjects with homozygous variations at all sites or a heterozygous variation at only one site. The diplotype configurations (a combination of haplotypes) were inferred by an expectation-maximization based program LDSUPPORT software, which determines the

posterior probability distribution of the diplotype for each subject based on the estimated haplotype frequencies.[14] Haplotypes without any amino acid change were designated as **1*, and the nonsynonymous variation-bearing haplotypes were numerically numbered. Subtypes were named with small alphabetical letters in the order of their frequencies. The ambiguous haplotypes inferred in only one subjects are grouped as "*others*" (**1* group) or shown as the haplotype name and a question mark (*NFE2L2*, **3a*).

Results and Discussion

As for *NFE2L2*, 14 genetic variations were found, including 9 novel ones, from 84 Japanese subjects (**Table 3**). Of these variations, 7 were located in the 5′-flanking region, 1 in the 5′-UTR, 3 (1 synonymous and 2 nonsynonymous) in the coding exons, 1 in the intron, and 2 in the 3′-UTR. All detected variations were in Hardy-Weinberg equilibrium ($p \geq 0.05$). Two novel nonsynonymous variations, 697C>T (Pro233Ser) and 1094G>T (Ser365Ile), were found as heterozygous variations. The allele frequencies were 0.012 for Pro233Ser and 0.006 for Ser365Ile. Nrf2 protein has 6 Nrf2-ECH homology (Neh) domains.[3] Pro233Ser is located between the Neh5 (amino acids 180–209) and Neh6 (338–388) domains, and Ser365Ile is within the Neh6 domain.[15,16] Since Neh6 domain is important for degradation of activated Nrf2 proteins, it is possible that Ser365Ile is functionally significant. This hypothesis should be clarified in future studies. Four known polymorphisms, −769G>A, −767G>A, and −733C>A in the 5′-flanking region and −89_−87delGCC in the 5′-UTR, were detected with allele frequencies similar to those previously reported in a Japanese population.[12] Of them, the number of −769G alleles and −733C/C genotype were reported to correlate with mononuclear cell infiltration to mucosa in chronic gastritis in Japanese,[17] and thus both variations may influence the expression of Nrf2 protein.

Regarding *KEAP1*, 18 genetic variations were detected, including 13 novel ones (**Table 4**): 2 were located in the 5′-flanking region, 4 in the coding exons (4 synonymous), 5 in the introns, 4 in the 3′-UTR, and 3 in the 3′-flanking region. All detected variations were in Hardy-Weinberg equilibrium ($p \geq 0.05$), except for three linked variations: IVS2-12delC, 2331 (*456)C>A, and 2407 (*532)+142C>T. These deviations were probably caused by the relatively small number of subjects analyzed. Since no nonsynonymous variation was found in *KEAP1* in this study, the sequences of this gene were well conserved and would be important for survival as observed in Keap1-deficient mice which died postnatally.[18] Gly364Cys previously found in two human lung cancer cell lines was shown to reduce the interaction with Nrf2,[19] but was not detected in this study.

Table 3. *NFE2L2* variations detected in this study

SNP ID			Location	Position		Nucleotide change	Amino acid change	Frequency
This Study	dbSNP (NCBI)	Reference		NT_005403.16	From the translational initiation site or from the end of the nearest exon			
MPJ6_2L2001[a]			5′-flanking	28339845	−1123	CGTTGATTCCACA>GGCATTTAATATA		0.190
MPJ6_2L2002	rs35652124	(12)	5′-flanking	28339491	−769	GGAGTTCAGAGGG>AGGGCGTTCAGGG		0.429
MPJ6_2L2003	rs6706649	(12)	5′-flanking	28339489	−767	AGTTCAGAGGAGG>AGCGTTCAGGGTG		0.048
MPJ6_2L2004[a]			5′-flanking	28339465	−743	GACTGCGAACACG>CAGCTGCCGGAGC		0.006
MPJ6_2L2005	rs6721961	(12)	5′-flanking	28339455	−733	ACGAGCTGCCGGC>AGCTGTCCACATC		0.321
MPJ6_2L2006[a]			5′-flanking	28339160_28339159	−438_−437	GCAAACAACTCT/del TT/ATCTCGCGGGCG		0.006
MPJ6_2L2007[a]			5′-flanking	28338860	−138	ATTACCGAGTGCC>TGGGGAGCCCGGA		0.012
MPJ6_2L2008		(12)	5′-UTR	28338811_28338809	−89_−87[b]	GCCGCCGCCGCC/del GCC/ACCAGAGCCGCC		0.589
MPJ6_2L2009[a]			Intron 2	28308078	IVS2+73	ATTAAAATATCTG>AGATTTGAGTTCC		0.018
MPJ6_2L2010[a]			Exon 3	28307426	372[b]	GCAGCTTTTGGCG>ACAGACATTCCCG	Ala124Ala	0.006
MPJ6_2L2011[a]			Exon 5	28306052	697[b]	TACTCATCTATAC>TCCTCAATGGAAA	Pro233Ser	0.012
MPJ6_2L2012[a]			Exon 5	28305655	1094[b]	TGGAATCTTCCAG>TCTATGGAGACAC	Ser365Ile	0.006
MPJ6_2L2013	rs34012004		3′-UTR	28304520	2229[b] (*411[c])	TATATTAAATTGT>GTTAGCTCTGGCA		0.071
MPJ6_2L2014[a]			3′-UTR	28304460_28304459	2289_2290[b] (*471_*472[c])	GATTATTATGAC/del TG/TTAAATTATTTG		0.006

[a] Novel variations detected in this study.
[b] Positions in cDNA (NM_006164.2).
[c] Positions are shown as * and bases from the translational termination codon TAG.

Table 4. *KEAP1* variations detected in this study

SNP ID		Location	Position		Nucleotide change	Amino acid change	Frequency
This Study	dbSNP (NCBI)		NT_011295.10	From the translational initiation site or from the end of the nearest exon			
MPJ6_KP1001[a]		5′-Flanking	1876957	−3446	AGTGAGAAGGGGG>CGCCTGGCTGTGC		0.006
MPJ6_KP1002[a]		5′-Flanking	1876866	−3355	GCCGCTCCTTTCC>GCGCCGCGCCCTT		0.036
MPJ6_KP1003[a]		Intron 1	1876599	−3088	CACCCCGAGGGAC>GCCCCTACGGAGG		0.036
MPJ6_KP1004	rs10412246	Intron 1	1875993	−2482	CGGCGCAACTCTT>CGGGGTGGCCCGG		0.030
MPJ6_KP1005[a]		Exon 2	1873422	90[b]	GGCAGGGGACGCG>AGTGATGTACGCC	Ala30Ala	0.006
MPJ6_KP1006[a]		Intron 2	1872723	IVS2+150	GTCTCCACAGTTT>AGAGGGGCTGAGG		0.006
MPJ6_KP1007[a]		Intron 2	1872660	IVS2+213	CAGGCAACATAAT>AGACACCCTGTCT		0.030
MPJ6_KP1008[a]		Intron 2	1865752	IVS2−12	CCGTCCCACTGT/del C/GCCCTCTGCAGG		0.048
MPJ6_KP1009	rs1048290	Exon 4	1863244	1413[b]	CCTCAATCGTCTC>GCTTTATGCCGTG	Leu471Leu	0.482
MPJ6_KP1010[a]		Exon 5	1862836	1542[b]	AGGCGTCTGCGTC>TCTGCACAACTGT	Val514Val	0.006
MPJ6_KP1011	rs11545829	Exon 5	1862767	1611[b]	CGTGGAGCGCTAC>TGATGTGGAAACA	Tyr537Tyr	0.315
MPJ6_KP1012[a]		3′-UTR	1859884	2121[b] (*246[c])	GGAAAGAGCAGGC>TTTCCAGGAGAGA		0.036
MPJ6_KP1013[a]		3′-UTR	1859681	2324[b] (*449[c])	CACTTCCCCACCG>AGATGGACAGTTA		0.036
MPJ6_KP1014[a]		3′-UTR	1859674	2331[b] (*456[c])	CCACCGGATGGAC>AAGTTATTTTGTT		0.036
MPJ6_KP1015	rs3177696	3′-UTR	1859613	2392[b] (*517[c])	AACAGACTAACTA>GGTGTCTTTCACC		0.018
MPJ6_KP1016	rs9676881	3′-flanking	1859582	2407 (*532)+16[d]	CTGGGCTGGAGGC>TCTGAAACCGGGG		0.506
MPJ6_KP1017[a]		3′-flanking	1859456	2407 (*532)+142[d]	CATCTTTCTCCCC>TGTAGGCAAGCCA		0.036
MPJ6_KP1018[a]		3′-flanking	1859441	2407 (*532)+157[d]	AGGCAAGCCACAC>TAGTTGTCCTTCA		0.024

[a] Novel variations detected in this study.
[b] Positions in cDNA (NM_203500.1).
[c] Positions are shown as * and bases from the translational termination codon TGA.
[d] Positions are shown as 2407 (*532) (final base of exon 6)+bases from the end of exon 6.

With the detected variations with ≥0.03 frequencies, linkage disequilibrium (LD) was analyzed using |D′| and r^2 values (data not shown). As for 6 variations in *NFE2L2*, 14 out of 15 (93%) combinations showed values of ≥0.99 in |D′|. In the r^2 values, strong linkage (r^2=0.93) was observed between −769G>A and −89_−87delGCC, and both variations were moderately linked with −733C>A (r^2≥0.63). Regarding *KEAP1*, 56 out of 66 (85%) combinations of 12 variations had values of ≥0.90 in |D′|. In the r^2 values, perfect linkages (r^2=1) were observed among −3355C>G, 2121 (*246)C>T and 2324 (*449)G>A, between −2482T>C and IVS2+213T>A, and between 2331 (*456)C>A and 2407 (*532)+142C>T. Strong linkage was found between 1413C>G (Leu471Leu) and 2407 (*532)+16C>T (r^2=0.82). Since relatively strong LDs were observed throughout the analyzed regions for both genes, haplotype analysis was performed as a single LD block for each gene (**Table 5 and 6**). The haplotypes detected on more than 5 chromosomes (3% in frequency) are called 'common' haplotypes in this paper.

As for *NFE2L2*, five haplotypes were first unambiguously assigned by homozygous variations at all sites (**1a* to **1c*) or a heterozygous variation at only one site (**1e*, and **1h*). Separately, the diplotype configurations (a combination of haplotypes) for all 84 subjects were estimated with over 0.95 certainty by LDSUPPORT software, except for four subjects. The additionally inferred haplotypes were seven **1*, one **2* (with 697C>T, Pro233Ser), and one **3* (with 1094G>T, Ser365Ile) haplotypes. The determined/inferred haplotypes are summarized in **Table 5**. The most frequent haplotype was **1a* (frequency: 0.375), followed by **1b* (0.292), **1c* (0.113), **1d* (0.071), **1e* (0.042), and **1f* (0.042). These 6 common haplotypes (**1a* to **1f*) accounted for 93% of all inferred haplotypes. Genotyping of the 5 haplotype-tagging variations, −1123A>G, −769G>A, −767G>A, −733C>A, and 2229 (*411)T>G, will discriminate these 6 common haplotypes.

Regarding *KEAP1*, seven haplotypes were unambiguously assigned by homozygous variations at all sites (**1a* to **1c*) or a heterozygous variation at only one site (**1d*, **1f*, **1g*, and **1j*). From the software analysis, the diplotype configurations for all 84 subjects were estimated with probability over 0.95. The additionally inferred haplotypes were eleven **1* subtypes. The summary of the determined/inferred haplotypes is shown in **Table 6**. The most frequent haplotype was **1a* (frequency: 0.417), followed by **1b* (0.310), **1c* (0.089), **1d* (0.030), and **1e* (0.030). These 5 common haplotypes accounted for 88% of all inferred haplotypes. To distinguish these 5 haplotypes, the 4 haplotype-tagging variations, −3088C>G, 1413C>G (Leu471Leu), 1611C>T (Tyr537Tyr), and 2324 (*449)G>A, can be

Table 5. *NFE2L2* Haplotypes

Haplotype[a,c]	Nucleotide change[a]	-1123A>G	-769G>A	-767G>A	-733C>A	-138C>T	-89_-87 delGCC	IVS2+73 G>A	697C>T	1094G>T	2229 (*411) T>G	Number	Frequency
	Amino acid change								Pro233Ser	Ser365Ile			
*1	*1a						*1a*					63	0.375
	*1b		*1b*		*1b*							49	0.292
	*1c	*1c*					*1c*					19	0.113
	*1d	*1d*					*1d*				*1d*	12	0.071
	*1e		*1e*	*1f*								7	0.042
	*1f		*1f*	*1f*			*1g*	*1g*				7	0.042
	*1g		*1g*			*1h*						3	0.018
	*1h		*1h*		*1h*	*1h*						1	0.006
	*1 others[d]											4	0.024
*2	*2a		*2a*		*2a*				*2a*			2	0.012
*3	*3a?[e]		*3a*	*3a*						*3a*		1	0.006
											Total	168	1.000

[a] Positions are numbered as described in **Table 3**. Haplotype-tagging variations for the common haplotypes **1a*-**1f* are underlined.
[b] Major allele, white; minor allele, gray with haplotype name.
[c] Haplotypes are described as numbers plus small alphabetical letters.
[d] Ambiguous **1* haplotypes inferred in only one subject are grouped into "*others*", and variations found only in these ambiguous haplotypes are not shown.
[e] The haplotype was inferred in only one subject and concurrent variations are ambiguous.

Table 6. *KEAP1* Haplotypes

Haplotype[a,c]	Nucleotide change[a]	-3355 C>G	-3088 C>G	-2482 T>C	IVS2+213 T>A	IVS2-12 delC	1413C>G	1611C>T	2121 (*246) C>T	2324 (*449) G>A	2331 (*456) C>A	2392 (*517) A>G	2407 (*532) +16C>T	2407 (*532) +142C>T	2407 (*532) +157C>T	Number	Frequency
	Amino acid change						Leu471Leu	Tyr537Tyr									
*1	*1a															70	0.417
	*1b						*1b*	*1b*					*1b*			52	0.310
	*1c						*1c*						*1c*			15	0.089
	*1d		*1d*													5	0.030
	*1e	*1e*							*1e*	*1e*			*1e*			5	0.030
	*1f														*1f*	4	0.024
	*1g			*1h*	*1h*	*1h*	*1g*					*1g*	*1g*			3	0.018
	*1h						*1h*				*1h*		*1h*	*1h*		3	0.018
	*1i					*1i*	*1i*				*1i*		*1i*	*1i*		2	0.012
	*1j												*1j*			1	0.006
	*1 others[d]															8	0.048
															Total	168	1.000

[a] Positions are numbered as described in **Table 4**. Haplotype-tagging variations for the common haplotypes *1a-*1e are underlined.
[b] Major allele, white; minor allele, gray with haplotype name.
[c] Haplotypes are described as numbers plus small alphabetical letters.
[d] Ambiguous *1 haplotypes inferred in only one subject are grouped into "*others*", and variations found only in these ambiguous haplotypes are not shown.

used.

In conclusion, 14 genetic variations including 9 novel ones, and 18 variations including 13 novels were detected in *NFE2L2* and *KEAP1*, respectively, in Japanese subjects. Using the detected variations, 14 (*NFE2L2*) and 18 (*KEAP1*) haplotypes were inferred, and 5 (*NFE2L2*) and 4 (*KEAP1*) haplotype-tagging variations were sufficient for identifying common haplotypes found at ≥ 0.03 frequencies. Since we analyzed type 2 diabetic patients, the frequencies of the alleles and haplotypes might be different from those in the healthy Japanese subjects. The allele frequencies of major variations in both genes, however, were comparable to those of healthy Japanese subjects in the previous studies [12, dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/)]. Thus, these data would be fundamental and useful information for pharmacogenetic studies on Nrf2-regulated genes for xenobiotic metabolism and disposition, many of which are involved in protection from oxidative stress.

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