a) Block 1

Nucleotide change	-609C>T	-477T>G	-266C>A	-243G>A	29C>A	85T>C	IVS2+158 T>C	IVS3+23 A>G	325T>A	451A>G	474T>C	IVS5-115G>A	496A>G	639C>T	733A>T	IVS7+64 G>T	793G>A	IVS8+91 C>T	IVS9-120A>T	1003G>T	IVS10+34 A>G	IVS10-15T>C	Number	Frequency
Amino acid change					A10E	C29R			Y109N	N151D	F153F		M166V	D213D	I245F		E265K			V335L				
Haplotype *1	*1a																						558	0.818
	*1b																						31	0.045
	*1c																						14	0.021
	*1d																						13	0.019
	*1e																						6	0.0088
	*1f																						6	0.0088
	*1g																						3	0.0044
	*1h																						2	0.0029
	*1i																						2	0.0029
	*1j																						2	0.0029
	*1k																						1	0.0015
	*1l																						1	0.0015
	*9	*9a					9																15	0.022
		*9d					9						9d										4	0.0059
		*9e§					9																1	0.0015
	*11	*11a																		11			1	0.0015
	*166V	*166Va											166V										8	0.012
		*166Vb											166V										3	0.0044
	*151D	*151D								151D													6	0.0088
	*109N	*109N							109N														2	0.0029
	*10E	*10E			10E																		1	0.0015
	*265K	*265K															265K						1	0.0015
	*245F	*245F§													245F								1	0.0015

b) Block 2

Nucleotide change			IVS12-11G>A	IVS12-9A>G	1543G>A	1572T>G	1627A>G	1666A>C	IVS13+39 C>T	IVS13+40 G>A	Number	Frequency
Amino acid change					V515I	F524L	I543V	S556R				
Haplotype	*1	*1a									361	0.529
		*1b									120	0.176
		*1c									5	0.0073
	*5	*5a					5				167	0.245
		*5b					5				26	0.038
	*515I	*515I			515I						1	0.0015
	*524L	*524L				524L					1	0.0015
	*556R	*556R§						556R			1	0.0015

c) Block 3

Nucleotide change			IVS13-47_48insTA	1752A>G	1774C>T	1896T>C	IVS14+19 C>A	IVS14+100 T>G	Number	Frequency
Amino acid change				T584T	R592W	F632F				
Haplotype	*1	*1a							578	0.848
		*1b							94	0.138
		*1c							6	0.0088
		*1d							1	0.0015
		*1e							1	0.0015
		*1f§							1	0.0015
	*592W	*592W			592W				1	0.0015

d) Block 4

Nucleotide change			IVS14-123C>A	IVS14-21C>A	IVS15+75 A>G	Number	Frequency
Amino acid change							
Haplotype	*1	*1a				576	0.845
		*1b				105	0.154
		*1c§				1	0.0015

e) Block 5

Nucleotide change			IVS16-127A>G	IVS16-94G>T	IVS17+34del T	IVS17+47C>T	2194G>A	Number	Frequency
Amino acid change							V732I		
Haplotype	*1	*1a						414	0.607
		*1b						255	0.374
		*1c§						1	0.0015
		*1d§						1	0.0015
		*1e§						1	0.0015
	*6	*6a					6	10	0.015

f) Block 6

Nucleotide change			IVS18-39G>A	2303C>A	2424T>C	2678A>G	IVS21+80 C>G	IVS21+113 T>A	IVS21+136 G>C	IVS21+162 T>G	IVS22+129 A>G	IVS22-69 G>A	IVS22-58 G>C	Number	Frequency
Amino acid change				T768K	S808S	N893S									
Haplotype	*1	*1a												624	0.915
		*1b												22	0.032
		*1c												5	0.0073
		*1d												2	0.0029
		*1e												2	0.0029
		*1f												2	0.0029
		*1g												2	0.0029
		*1h												1	0.0015
		*1i												1	0.0015
	*768K	*768K		768K										19	0.028
	*893S	*893S				893S								2	0.0029

Fig. 3 Block haplotypes in *DPYD* of block 1 (**a**), block 2 (**b**), block 3 (**c**), block 4 (**d**), block 5 (**e**), and block 6 (**f**) in a Japanese population. The nucleotide positions were numbered based on the cDNA sequence (A of the translational start codon is +1) or from the nearest exon. *White cell* wild-type, *gray cell* nucleotide alteration. §The haplotypes were inferred in only one patient and ambiguous except for marker SNPs

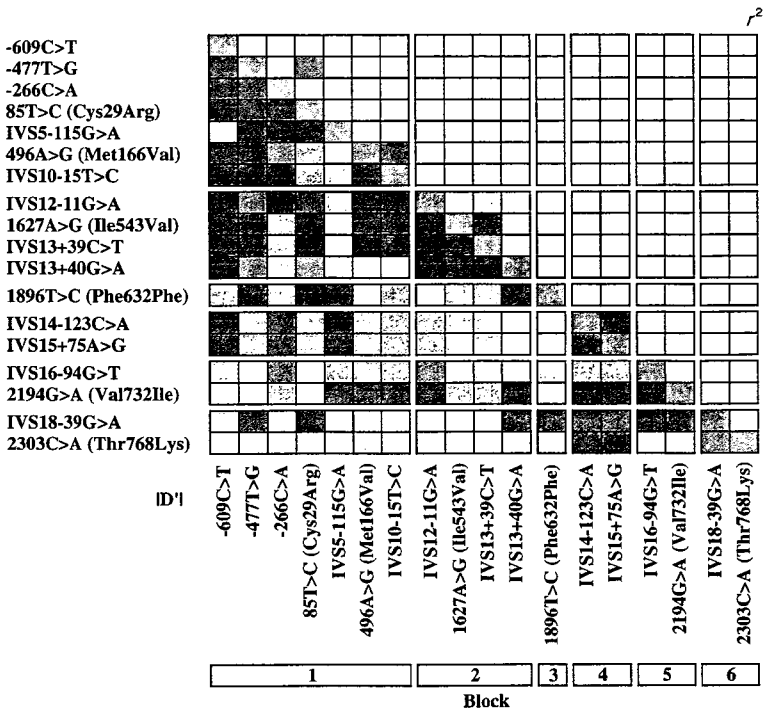

Fig. 2 Linkage disequilibrium (*LD*) analysis of *DPYD*. Pairwise LD between 18 common SNPs (>0.01 in allele frequencies) is expressed as r^2 (*upper*) and |D'| (*lower*) by a 10-graded *blue color*. The *denser color* indicates higher linkage. The haplotype block partition based on LD measure |D'| of HapMap data in Japanese is also indicated

or HapMap project. Notably, IVS14 + 1G>A (*2), 1897delC (Pro633GlnfsX5, *3), 1601G>A (Ser534Asn, *4), 295_298delTCAT (Phe100SerfsX15, *7), 703C>T (Arg235Trp, *8), 2983G>T (Val995Phe, *10), 62G>A (Arg21Gln, *12), 1156G>T (Glu386X, *12), and 1679T>G (Ile560Ser, *13) were not found in this study. Furthermore, several SNPs showed marked differences in allele frequencies among Japanese and other ethnic groups (Table 4).

The allele frequency of 85T>C (Cys29Arg, *9), the tagging SNP for block 1 #9, was quite different between Asians and Caucasians. Its allele frequency in Japanese (0.029 in this study) and Taiwanese (0.022) (Hsiao et al. 2004) was much lower than that in Caucasians (0.185–0.194) (Seck et al. 2005; Morel et al. 2006).

The SNP 496A>G (Met166Val) in block 1 is found at a lower allele frequency in Japanese (0.022) than in Caucasians (0.080) (Seck et al. 2005). Seck et al. (2005) inferred two haplotypes harboring 496A>G (Met166Val) from 157 Caucasians: *hap5* (#9d in this study) harboring additional 85T>C (Cys29Arg) and IVS10-15T>C and *hap11* concurrently harboring IVS10-15T>C alone with frequencies of 0.040 and 0.014, respectively. In our haplotype analysis, #166Va (0.012) corresponding to *hap11* (0.014) was found with a similar frequency in Japanese, whereas the frequency of #9d (0.006) was much lower than that of the corresponding haplotype, *hap5* (0.040) in Caucasians.

1627A>G (Ile543Val, *5) in block 2 was found with comparable allele frequencies among Japanese (0.283 in this study), Caucasians (0.14-0.275) (Seck et al. 2005; Ridge et al. 1998a), African-Americans (0.227) (Wei et al. 1998), and Taiwanese (0.210–0.283) (Wei et al. 1998; Hsiao et al. 2004).

The allele frequency (0.015) of 2194G>A (Val732Ile, *6) in block 5 in our Japanese population is slightly lower than that previously reported in Caucasians (0.022-0.058) (Seck et al. 2005; Ridge et al. 1998a) and Finish (0.067) (Wei et al. 1998), but is comparable to that in Taiwanese (0.012-0.014) (Wei et al. 1998; Hsiao et al. 2004) and African-Americans (0.019) (Wei et al. 1998).

Ethnic differences in the allele frequencies were also observed with synonymous and intronic variations (Table 4). The allele frequency of 1896T>C (Phe632Phe), which tags block 3 #1b, was higher in Japanese (0.139 in this study) than in Caucasians (0.035) (Seck et al. 2005). *Hap13* assigned in 157 Caucasians by Seck et al. (2005) is the counterpart of block 3 #1b, and its frequency (0.012) was much lower than that in Japanese (0.138).

In contrast, IVS10-15T>C linked to 85T>C (*9) or 496A>G (#166V) within block 1 showed a lower allele frequency in Japanese (0.018) than in Caucasians (0.127). Seck et al. (2005) assigned *hap7* as the haplotype containing IVS10-15T>C alone with a haplotype frequency of 0.03 in Caucasians. In Japanese, however, the corresponding haplotype was not found.

Allele frequencies of IVS18-39G>A and IVS22-69G>A, which are tagging SNPs for block 6 #1b and #1f, respectively, are lower in Japanese (0.032 and 0.003, respectively) than in Caucasians (0.105 and 0.183, respectively).

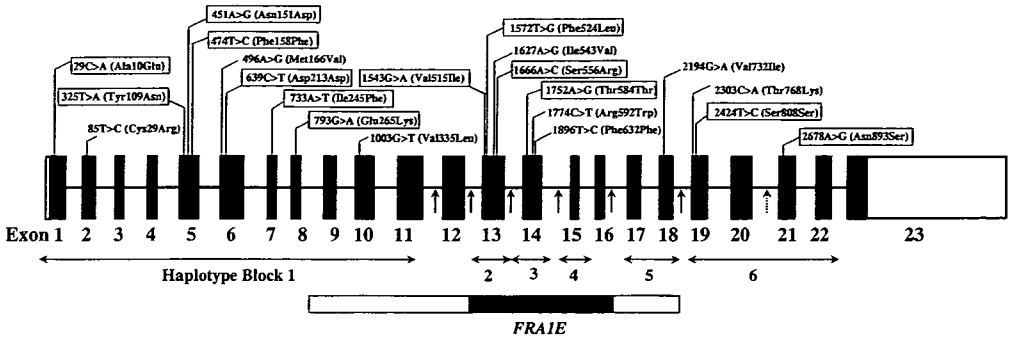

Fig. 1 Twenty-one variations detected in the coding exons are depicted in the schematic diagram of the *DPYD* gene. Fourteen novel variations are enclosed by *squares*. The recombination spots were estimated based on the LD profiles obtained from Japanese data in the HapMap project and indicated by *arrows*. The borders (between introns 8 and 18 of the *DPYD*) and core region (between introns 12 and 16) of *FRA1E* identified by Hormozian et al. (2007) are indicated as an *open* and *closed box*, respectively

In block 2, four haplotypes, #*1a* (0.529), #*5a* (0.245), #*1b* (0.176), and #*5b* (0.038), were major in Japanese and accounted for 99% of all inferred haplotypes. Two subtypes of the #*5* group, #*5a* and #*5b*, both of which harbored Ile543Val (**5*) and IVS13 + 39C>T, were distinguished by a novel intronic SNP, IVS12-11G>A.

As for block 3, in addition to #*1a* (0.848), #*1b* harboring the synonymous SNP, 1896T>C (Phe632Phe), was found at a relatively high frequency (0.138).

Block 4 is simple and comprises only three haplotypes, #*1a* (0.845), #*1b* (0.154) and #*1c* (0.0015). The second frequent haplotype, #*1b*, harbored perfectly linked SNPs, IVS14-123C>A and IVS15 + 75A>G.

Block 5 contained IVS16-94G>T, the most frequent SNP among the 55 SNPs found in this study, which was assigned to #*1b* with a frequency of 0.374. This block also contained the known nonsynonymous SNP, 2194G>A (Val732Ile, **6*), which was assigned to #*6a* (0.015).

In block 6, the most dominant haplotype was #*1a* (0.915). It was followed by #*1b* (0.032) with IVS18-39G>A and #*768K* (0.028) with 2303C>A (Thr768Lys).

The HapMap data include nine SNPs that we detected (Table 2). Of them, six, 85T>C (rs1801265), 496A>G (rs2297595), 1627A>G (rs1801159), 1896T>C (rs17376848), IVS16-94G>T (rs7556439) and IVS18-39G>A (rs12137711), were suitable for haplotype tagging SNPs (htSNPs) to capture the block haplotypes, block 1 #*9*, block 1 #*166V*, block 2 #*5*, block 3 #*1b*, block 5 #*1b*, and block 6 #*1b*, respectively. IVS21 + 136G>C (rs11165777) and IVS22-69G>A (rs290855)/IVS22-58G>C (rs1711 6357), were the marker SNPs for block 6 #*1e* and #*1f*, respectively, but very rare (allele frequencies = 0.003) in Japanese. The six SNPs, especially 85T>C (rs1801265) and 496A>G (rs2297595), were in strong LD ($r^2 > 0.8$) with other HapMap SNPs in Japanese (Table 3), indicating that many HapMap SNPs were concurrently linked on the same haplotypes.

Next, the combinations of block haplotypes (inter-block haplotypes) were analyzed focusing on the haplotypes with frequencies of >0.01 in each block (Fig. 4). Between blocks 1 and 2, both #*1a* and #*1b* in block 1 were complicatedly associated with various haplotypes in block 2. It should be noted that #*9c* in block 1 was linked either with block 2 #*1b* (0.016 in absolute frequency) or with block 2 #*5a* (0.006, not shown in Fig. 4). #*1c* in block 1 was completely linked with block 2 #*1a*. #*151D* in block 1 (not shown in Fig. 4), which was a rare haplotype (0.009) harboring 451A>G (Asn151Asp), was completely linked with #*5a* in block 2.

Between blocks 2 and 3, both #*5b* and #*1b* in block 2 were mostly linked with #*1a* in block 3, whereas both #*1a* and #*5a* in block 2 were complicatedly linked with #*1a*, #*1b*, or other rare haplotypes such as #*1c* (not shown in Fig. 4) in block 3. Between blocks 3 and 4 and between blocks 4 and 5, no strong associations of block haplotypes were observed except for the linkage of block 5 #*6a* to block 4 #*1a*. Between blocks 5 and 6, most of #*1b* and all of #*6a* in block 5 were linked with #*1a* in block 6. Although #*1a* in block 6 was associated with various haplotypes in block 5, #*1b* in block 6 was completely linked with #*1a* in block 5.

Among the six blocks, the following combinations were major: #*1a* (block 1)–#*1a* (block 2) –#*1a* (block 3)–#*1a* (block 4)–#*1a* (block 5)–#*1a* (block 6) (0.239 in frequency), #*1a*–#*5a*–#*1a*–#*1a*–#*1b*–#*1a* (0.081), #*1a*–#*1a*–#*1a*–#*1a*–#*1b*–#*1a* (0.075), #*1a*–#*5a*–#*1a*–#*1a*–#*1a*–#*1a* (0.070), #*1a*–#*1b*–#*1a*–#*1a*–#*1a*–#*1a* (0.060) and #*1a*–#*1a*–#*1b*–#*1a*–#*1a*–#*1a* (0.051).

Ethnic differences in distributions of *DPYD* SNPs and haplotypes

We compared SNP and haplotype distributions in Japanese with those in other ethnic groups reported in the literature

In the 5′ flanking region, all four detected SNPs (-609C>T, -477T>G, -266C>A, -243G>A) were newly found at relatively high allele frequencies (0.006–0.05). However, these SNPs were not located near the proposed *cis*-regulatory promoter elements (Shestopal et al. 2000). The remaining 21 novel variations were found in intronic regions. Of these SNPs, IVS5–115G>A, IVS12–11G>A, and IVS14-123C>A were detected with allele frequencies of 0.021, 0.038, and 0.155, respectively, but others were rare (<0.01). They were not located in the exon-intron splicing junctions or branch sites.

Seventeen variations were already reported. The ID numbers in the dbSNP databases or references for these SNPs are described in Table 2. The well-known nonsynonymous SNPs, 1627A>G (**5*, Ile543Val), 2194G>A (**6*, Val732Ile), 85T>C (**9*, Cys29Arg), and 1003G>T (**11*, Val335Leu), were found in this study at allele frequencies of 0.283, 0.015, 0.029, and 0.0015, respectively. The allele frequencies of two reported SNPs, 496A>G (Met166Val) and 2303C>A (Thr768Lys), were 0.022 and 0.028, respectively. Recently, 1774C>T (Arg592Trp) was reported from a Korean population (Cho et al. 2007), and its allele frequency was 0.0015 in this study. Nine intronic variations, IVS10-15T>C, IVS13 + 39C>T, IVS13 + 40 G>A, IVS15 + 75A>G, IVS16-94G>T, IVS18-39G>A, IVS21 + 136G>C, IVS22-58G>C, and IVS22-69G>A, and one synonymous variation, 1896T>C (Phe632Phe), were found with various allele frequencies (0.003–0.378, Table 2). The variations previously detected in Japanese (Kouwaki et al. 1998; Yamaguchi et al. 2001; Ogura et al. 2005), 62G>A (Arg21Gln, **12*), 74G>A (His25Arg), 812delT (Leu271X), 1097G>C (Gly366Ala), 1156G>T (Glu386X, **12*), and 1714C>G (Leu572Val), were not found in our study. This might be due to their low frequencies.

Linkage disequilibrium (LD) analysis and haplotype block partition

LD analysis was performed by r^2 and |D′| using 18 SNPs (allele frequency ≥0.01) (Fig. 2). Strong linkages were observed in four pairs of SNPs: between -477T>G and 85T>C (Cys29Arg) (r^2 = 0.7025), between 496A>G (Met166Val) and IVS10-15T>C (r^2 = 0.7964), between 1627A>G (Ile543Val) and IVS13 + 39C>T (r^2 = 1.0), and between IVS14-123C>A and IVS15 + 75A>G (r^2 = 1.0). In addition, two known rare SNPs, IVS22-69G>A (rs290855) and IVS22-58G>C (rs17116357), were perfectly linked (r^2 = 1.0) (data not shown). As for |D′| values, only 43 pairs (28%) out of 153 pairs gave |D′| = 1.0, indicating that a number of recombinations had occurred within this gene. This is not surprising because *DPYD* is a huge gene of at least 950 kb in length with 3 kb of coding sequences. However, it was difficult to estimate past recombination events in *DPYD* from our data alone because our variations were mostly limited to exons and surrounding introns.

To define haplotype blocks, we utilized the HapMap data because SNPs were comprehensively genotyped with an average density of 1 SNP per 1.8 kb. Of 1,002 variations of *DPYD* genotyped by the HapMap project, 474 SNPs were polymorphic for 44 unrelated Japanese subjects. When the LD profiles for Japanese were obtained by Marker using the HapMap data, strong LD (|D′| > 0.75) clearly decays within introns 11, 12, 13, 14, 16, 18, and 20 (data not shown), suggesting that recombination had occurred in these regions. Based on these findings, the SNPs detected in our study were divided into six haplotype blocks (Figs. 1, 2). Block 1, the largest block, ranges from the 5′-untranslated region (5′-UTR) to intron 10 (347 kb), and includes 22 variations. Block 2 includes eight variations from IVS12-11G>A in intron 12 to IVS13 + 40G>A in intron 13. Block 3 includes six variations from IVS13-47_48insTA in intron 13 to IVS14 + 100T>G in intron 14. Block 4 contains only three SNPs, IVS14-123C>A, IVS14-21C>A and IVS15 + 75A>G, and ranges from intron 14 to intron 15. Block 5 consists of IVS16-94G>T and four rare variations from intron 16 to exon 18. Although the HapMap data showed a decline in LD in intron 20, we defined a block ranging from intron 18 to intron 22 as block 6 because only rare variations (allele frequencies <0.01) were detected downstream of intron 20 (exon 21, intron 21, and intron 22). The block partitioning based on the HapMap data fitted our SNPs well: more than 70% of SNP pairs in each block (block 1–6) gave pair-wise |D′| values greater than 0.8 (Fig. 2).

Haplotype estimation

Using 22, 8, 6, 3, 5, and 11 variations in blocks 1 to 6, 23 (block 1), 8 (block 2), 7 (block 3), 3 (block 4), 6 (block 5), and 11 (block 6) haplotypes were identified or inferred (Fig. 3). Probabilities of diplotype configurations in all six blocks were 100% for over 97% of the subjects. To discriminate our block haplotypes from the previously assigned alleles or haplotypes (*DPYD*1* to **13*), the mark, #, was used to indicate block haplotypes.

In block 1, the most dominant haplotype without any variation was #*1a* (0.818 in frequency), followed by #*1b* (0.045), #*9c* (0.022), and #*1c* (0.021). As suggested by LD (Fig. 2), #*9c*, the major subtype of the #*9* group bearing 85T>C (Cys29Arg), also harbored -477T>G in the 5′-UTR. Known nonsynonymous SNP, 496A>G (Met166Val), was assigned to three haplotypes, #*9d*, #*166Va*, and #*166Vb*.

Table 2 continued

SNP ID		Location	Position		Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Reported alleles	Allele frequency (341 subjects)
This study	dbSNP (NCBI)		NT_032977.7	From the translational initiation site or from the end of nearest exon				
MPJ6_DPD041[g]	rs7556439	Intron 16	51591340	IVS16-94	CAAGTTGGATTTG/TTCTTGCACGTCT			0.378
MPJ6_DPD042[a]		Intron 17	51591092	IVS17 + 34	GTTGCCCGCTATT/-GTAAATATTGGC			0.0015
MPJ6_DPD043[a]		Intron 17	51591079	IVS17 + 47	GTAAATATTGGCC/TCACATTATGTAG			0.0015
MPJ6_DPD044	rs1801160	Exon 18	51590313	2194	GGTGCCAATGGCG/ATTACAGCCACCA	Val732Ile	*6	0.015
MPJ6_DPD045[g]	rs12137711	Intron 18	51519982	IVS18-39	TATACTCAAGTGG/ATCAGTGTGCTAA			0.032
MPJ6_DPD046[c]		Exon 19	51519940	2303	TTTGTGTAGGGAC/AAGCAATCAGACC	Thr768Lys		0.028
MPJ6_DPD047[a]		Exon 19	51519819	2424	GTTTCTCCATAGT/CGGTGCTTCCGTC	Ser808Ser		0.0029
MPJ6_DPD048[a]		Exon 21	51383526	2678	TCATAGCAGAAAA/GCAAGATTAGACT	Asn893Ser		0.0029
MPJ6_DPD049[a]		Intron 21	51383358	IVS21 + 80	GTTTATTTACTGC/GTTAAATGTATCA			0.0015
MPJ6_DPD050[a]		Intron 21	51383325	IVS21 + 113	GTTTTAGAATTAT/AAATGAAAGTTTT			0.0015
MPJ6_DPD051[g]	rs11165777	Intron 21	51383302	IVS21 + 136	TTAAAAACATCTG/CTCCATGGTGAAA			0.0029
MPJ6_DPD052[a]		Intron 21	51383276	IVS21 + 162	CTGCATTTAAATT/GATAAAATAACCT			0.0029
MPJ6_DPD053[a]		Intron 22	51367150	IVS22 + 129	TTCTGCAACAGTA/GCATCTTTCTGTC			0.0073
MPJ6_DPD054[g]	rs290855	Intron 22	51364164	IVS22-69	GAGAAAAATGTTG/AACGCTAAAATGG			0.0029
MPJ6_DPD055[g]	rs17116357	Intron 22	51364153	IVS22-58	TAACGCTAAAATG/CGGGACATTGTTG			0.0029

[a] Novel variations detected in this study

[b] Kouwaki et al. 1998

[c] Collie-Duguid et al. 2000

[d] Seck et al. 2005

[e] Ogura et al. 2005

[f] Cho et al. 2007

[g] Variations overlapping with the HapMap project

Table 2 Summary of *DPYD* SNPs detected in a Japanese population

SNP ID		Location	Position		Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Reported alleles	Allele frequency (341 subjects)
This study	dbSNP (NCBI)		NT_032977.7	From the translational initiation site or from the end of nearest exon				
MPJ6_DPD001[a]		5′-Flank	52206480	–609	TTGCTCGCCTCCC/TTCCCCTCCCCGC			0.021
MPJ6_DPD002[a]		5′-Flank	52206348	–477	TTGAGGAGTTCCT/GGAAAATGCAGTT			0.026
MPJ6_DPD003[a]		5′-Flank	52206137	–266	CTCCCTCCCTCCC/ATTCTGCTTGCAG			0.045
MPJ6_DPD004[a]		5′-Flank	52206114	–243	AGGCTGGGGCGCG/AGAGCGGGCTGAA			0.0059
MPJ6_DPD005[a]		Exon 1	52205843	29	GTAAGGACTCGGC/AGGACATCGAGGT	Ala10Glu		0.0015
MPJ6_DPD006[g]	rs1801265	Exon 2	52168278	85	CATGCAACTCTGT/CGTTCCACTTCGG	Cys29Arg	*9*	0.029
MPJ6_DPD007[a]		Intron 2	52168055	IVS2 + 158	TTTGAAAGTGTAT/CTTTTAATTACAC			0.0015
MPJ6_DPD008[a]		Intron 3	52113040	IVS3 + 23	GTCACCATAGCAA/GCAGTCACAGATG			0.0029
MPJ6_DPD009[a]		Exon 5	52006617	325	ATTTTGCAGAACT/AATTATGGAGCTG	Tyr109Asn		0.0029
MPJ6_DPD010[a]		Exon 5	52006491	451	GAGGGACCCATTA/GATATTGGTGGAT	Asn151Asp		0.0088
MPJ6_DPD011[a]		Exon 5	52006468	474	ATTGCAGCAATTT/CGCTACTGAGGTA	Phe158Phe		0.0044
MPJ6_DPD012[a]		Intron 5	51984611	IVS5–115	CATATTAATACTG/AAAAATGTACTGC			0.021
MPJ6_DPD013[g]	rs2297595	Exon 6	51984484	496	GTATTCAAAGCAA/GTGAGTATCCCAC	Met166Val		0.022
MPJ6_DPD014[a]		Exon 6	51984341	639	GGGGTACTCTGAC/TATCACTATATTT	Asp213Asp		0.0088
MPJ6_DPD015[a]		Exon 7	51976695	733	GTGAATTTTGAGA/TTTGAGCTAATGA	Ile245Phe		0.0015
MPJ6_DPD016[a]		Intron 7	51976602	IVS7 + 64	CTCTACACTAAAG/TATTAACAGCAAA			0.0015
MPJ6_DPD017[a]		Exon 8	51964101	793	CTTTCAGTGAATG/AAAATGACTCTTA	Glu265Lys		0.0015
MPJ6_DPD018[a]		Intron 8	51963953	IVS8 + 91	TTCAGACATTTTC/TGTGATGAAAGTT			0.0088
MPJ6_DPD019[a]		Intron 9	51878456	IVS9–120	TTTGATAGTGACA/TCTTCATCCTGGA			0.0029
MPJ6_DPD020[b]		Exon 10	51878292	1003	ATACGGGGAGTCG/TTGATTGTACTTG	Val335Leu	*11*	0.0015
MPJ6_DPD021[a]		Intron 10	51878143	IVS10 + 24	CCATCAGAAAAT A/GTGGAGTTGTACT			0.0015
MPJ6_DPD022[c]		Intron 10	51858934	IVS10-15	TTTCTTCTCTGTT/CCTGTTTTGTTTT			0.018
MPJ6_DPD023[a]		Intron 12	51800901	IVS12-11	AAGTATTGGTTTG/ATATTTTGCAGTC			0.038
MPJ6_DPD024[a]		Intron 12	51800899	IVS12-9	GTATTGGTTTGTA/GTTTTGCAGTCAC			0.0073
MPJ6_DPD025[a]		Exon 13	51800872	1543	TATGGAGCTTCCG/ATTTCTGCCAAGC	Val515Ile		0.0015
MPJ6_DPD026[a]		Exon 13	51800843	1572	ACTACCCCTCTTT/GTACACTCCTATT	Phe524Leu		0.0015
MPJ6_DPD027[g]	rs1801159	Exon 13	51800788	1627	GGATTGAAGTTTA/GTAAATCCTTTTG	Ile543Val	*5*	0.283
MPJ6_DPD028[a]		Exon 13	51800749	1666	ACTCCAGCCACCA/CGCACATCAATGA	Ser556Arg		0.0015
MPJ6_DPD029	rs2786783	Intron 13	51800636	IVS13 + 39	AGAAATGTCTATC/TATATATTTTAAT			0.283
MPJ6_DPD030	rs2811178	Intron 13	51800635	IVS13 + 40	GAAATGTCTATCG/ATATATTTTAATT			0.179
MPJ6_DPD031[a]		Intron 13	51735220_51735219	IVS13-47_-48	ATAAAGATTATA–/TAGCTTTTCTTTGT			0.0015
MPJ6_DPD032[a]		Exon 14	51735161	1752	GGACATTGTGACA/GAATGTTTCCCCC	Thr584Thr		0.0015
MPJ6_DPD033[f]		Exon 14	51735139	1774	CCCAGAATCATCC/TGGGGAACCACCT	Arg592Trp		0.0015
MPJ6_DPD034[g]	rs17376848	Exon 14	51735017	1896	AAAGGCTGACTTT/CCCAGACAACGTA	Phe632Phe		0.139
MPJ6_DPD035[a]		Intron 14	51734989	IVS14 + 19	GTGATTTAACATC/ATAAAACAAGAGA			0.0088
MPJ6_DPD036[a]		Intron 14	51734908	IVS14 + 100	TTAATGTGTATAT/GTTTATTAAAGAA			0.0015
MPJ6_DPD037[a]		Intron 14	51667533	IVS14-123	GATTTATTTTTTC/AACAGTTTGAAAA			0.155
MPJ6_DPD038[a]		Intron 14	51667431	IVS14-21	TGAACTTATATTC/ATTTTGTTTTTCT			0.0015
MPJ6_DPD039[d]		Intron 15	51667267	IVS15 + 75	TAAAGAGCTGCCA/GTGAGAAATAATA			0.155
MPJ6_DPD040[a]		Intron 16	51591373	IVS16-127	GGAATTTGAGAAA/GTATATCATGTAG			0.0015

Foster City, CA) with the primers listed in "sequencing" of Table 1. Excess dye was removed with a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All novel SNPs were confirmed by sequencing of PCR products generated from new genomic DNA amplifications. The genomic and cDNA sequences of *DPYD* obtained from GenBank (NT_032977.7 and NM_000110.2, respectively) were used as reference sequences. SNP positions were numbered based on the cDNA sequence, and adenine of the translational initiation site in exon 1 was numbered +1. For intronic polymorphisms, the position was numbered from the nearest exon.

Linkage disequilibrium (LD) and haplotype analyses

Hardy-Weinberg equilibrium and LD analyses were performed by SNPAlyze software (Dynacom Co., Yokohama, Japan), and pairwise LD parameters between variations were obtained as the |D'| and rho square (r^2) values. Some haplotypes were unambiguously identified from subjects with homozygous variations at all sites or a heterozygous variation at only one site. Diplotype configurations were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype for each subject based on the estimated haplotype frequencies (Kitamura et al. 2002). Although the nomenclature for nonsynonymous *DPYD* alleles (*DPYD*1* to *DPYD*13*) have been already publicized (McLeod et al. 1998; Collie-Duguid et al. 2000; Johnson et al. 2002), several reported alleles remain unassigned. To avoid confusion with the previous *DPYD* allele nomenclature, our block haplotypes in this study were tentatively defined by using '#' instead of '*'. A group of haplotypes without any amino acid change is designated as *#1*, and the haplotype groups bearing already defined alleles, *DPYD*5* (Ile543Val), *DPYD*6* (Val732Ile), *DPYD*9* (Cys29Arg) and *DPYD*11* (Val335Leu), were numbered by using the corresponding Arabic numerals, *#5*, *#6*, *#9*, and *#11*, respectively. Other haplotypes with known nonsynonymous SNPs such as 496A>G (Met166Val) or with the novel nonsynonymous SNP were represented by '#' plus amino acid positions followed by variant residues (for example, *#166V*). Subtypes within each haplotype group were consecutively named with small alphabetical letters depending on their frequencies. Haplotypes ambiguously inferred in only one patient were indicated in the Fig. 3 legend. Combinations of block haplotypes were analyzed by Haploview software (http://www.broad.mit.edu/mpg/haploview/index.php) (Barrett et al. 2005), and the long-range (whole gene) haplotypes spanning all blocks were inferred by Hapblock software (www.cmb.usc.edu/msms/HapBlock/) (Zhang et al. 2005).

Typing data on *DPYD* from unrelated 44 Japanese and 30 Caucasian trios were also obtained from the HapMap project (HapMap release 19: http://www.hapmap.org/). The LD profiles and haplotypes of the HapMap data were obtained by Marker beta in Gmap Net (http://www.gmap.net/marker) using its four (1254711, 1254712, 1254713, and 1254714) and six (1166276, 1166277, 1166278, 1166279, 1166280, and 1166281) datasets covering *DPYD* genomic regions for Japanese and Caucasians, respectively.

Drawing of protein structures

The coordinate data (1gth) of the crystal structure of pig DPD (Dobritzsch et al. 2002) was obtained from the Protein Data Bank. Protein Explorer (http://proteinexplorer.org) (Martz 2002) was used to display the structural features of pig DPD and depict three-dimensional views.

Results

DPYD variations found in a Japanese population

We identified 55 variations, including 38 novel ones by sequencing the promoter regions (up to 613 bp upstream from the translational initiation site), all 23 exons and their flanking regions of *DPYD* from 341 Japanese subjects (Table 2). The distribution of the variations consisted of 4 in the 5' flanking region, 21 (5 synonymous and 16 nonsynonymous ones) in the coding exons (Fig. 1) and 30 in the introns. Since we did not find any significant differences in allele frequencies between healthy volunteers and cancer patients ($P > 0.05$ by χ^2 test or Fisher's exact test) except for one variation, IVS14 + 19C>A, ($P = 0.027$ by Fisher's exact test); the data for all subjects were analyzed as one group. All detected variations except for 451A>G (Asn151Asp) and IVS13 + 40G>A were in Hardy-Weinberg equilibrium ($P \geq 0.24$).

Thirteen novel variations in the coding region (enclosed by a square in Fig. 1) contain four synonymous SNPs, 474T>C (Phe158Phe), 639C>T (Asp213Asp), 1752A>G (Thr584Thr), and 2424T>C (Ser808Ser) and nine nonsynonymous SNPs, 29C>A (Ala10Glu), 325T>A (Tyr109Asn), 451A>G (Asn151Asp), 733A>T (Ile245Phe), 793G>A (Glu265Lys), 1543G>A (Val515Ile), 1572T>G (Phe524-Leu), 1666A>C (Ser556Arg), and 2678A>G (Asn893Ser). 451A>G (Asn151Asp), 325T>A (Tyr109Asn), and 2678A>G (Asn893Ser) were found at frequencies of 0.009, 0.003 and 0.003, respectively. The others were detected as single heterozygotes (allele frequencies = 0.0015).

Table 1 Primer sequences for human *DPYD*

Amplified and sequenced region		Forward primer		Reverse primer		PCR product (bp)
		Sequences (5' to 3')	Position[a]	Sequences (5' to 3')	Position[a]	
	Exon 14	TGCAAATATGTGAGGAGGGACC	51735287	CAGCAAAGCAACTGGCAGATTC	51734877	411
	Exon 15	GCTATCTTACCCTGCTATTTTC	51667571	TAGGTAGTGTGTGAAATCCAAGG	51667107	465
	Exon 16	CCCCTTATGAGCACTGAGTAAAT	51658821	TAGTAACTATCCATACGGGGG	51658440	382
	Exon 17	AGTCTAGGTGTAATACTGAGGAGG	51591407	ATCAAGTGCTCAACTGGAAACT	51590986	422
	Exon 18	GTGAAGAACTTTGAGGAGAAGAC	51590461	CATCCTGTGCTGTCACTTGA	51590026	436
	Exon 19	ATTTGTCCAGTGACGCTGTC	51520048	TCAGGTCTCTTCATAACTTGTCAG	51519629	420
	Exon 20	GAGAAGTGAATTTGTTTGGAG	51478265	TTTGTTAGTGAGAATGTGAGATGG	51477926	340
	Exon 21	AGTGGTCCAAAACAATGAGTG	51383737	TGCTTGCCAGTGTTCTAAAA	51383221	517
	Exon 22	GGGTGTCATTTATTCTTTCTGTC	51367723	GGCTGATGAAATGGTATAAAAA	51367033	691
	Exon 23	GTTGTCTCATAGTGTGGCTCCTC	51364206	TTTTTCACATAAGACAACTGGCA	51363641	566
Sequencing	5'-UTR to exon 1	TGTGGATGTTTTTGCTCGC	52206503			
	5'-UTR to exon 1	CGGACTGCTTTTACCTTTGC	52206258	CCAGAGAGCCAAGTGACAGC	52205933	
	5'-UTR to exon 1	CCCTAGTCTGCCTGTTTTCG	52205987	AGTAAACAGGTCCCGACGC	52205586	
	Exon 2	GTGACAAAGTGAGAGAGACCGT	52168436	GCCTTACAATGTGTGGAGTGAG	52168152	
	Exon 3	GAATGCTACCCAATTAAAGTGG	52113285	TTCAAAACCAAATACAGCCTC	52112899	
	Exon 4	TGCCAAAGATGAAACACAGA	52025601	ACCCACAGATAATAGAGAACAAGA	52025273	
	Exon 5	TGATGGTTCCTGATAGTAGTATTG	52006775	TGTCACACTAAAAATGTTGGG	52006348	
	Exon 6	AAAATATGTTTGAGGATGTAAGC	51984560	GAGCCTGAAGTTCCTATATGAT	51984201	
	Exon 7	TTCTACTGTATCTTCACTCCACG	51976953	GCTTCTGCCTGATGTAGC	51976541	
	Exon 8	GGCTGACTTTTCATTCTTTTT	51964221	CATCTTGCCGAAATCTCTCC	51963831	
	Exon 9	TGTGATTTACGATGTGTACTTGG	51880335	GCAAGGTTGGGTGTGAGAG	51879895	
	Exon 10	AAAATGGGAATAAAACTGTCTT	51878507	TTCATCTCCTAAAATCTGTTGG	51878109	
	Exon 11	ACTGGTAACTGAAACTCAG	51859069	CAATTCCCTGAAAGCTAG	51858628	
	Exon 12	TCAGTGCCCTTCAAATGTGT	51834881	GAGTATCAAAAATAAATGAAGCAC	51834439	
	Exon 13	TCGGATGCTGTGTTGAAGTG	51800982	TGTGTAATGATAGGTCGTGTC	51800543	
	Exon 14	TGCAAATATGTGAGGAGGGACC	51735287	CAGCAAAGCAACTGGCAGATTC	51734877	
	Exon 15	GCTATCTTACCCTGCTATTTTC	51667571	TAGGTAGTGTGTGAAATCCAAGG	51667107	
	Exon 16	CCCCTTATGAGCACTGAGTAAAT	51658821	TAGTAACTATCCATACGGGGG	51658440	
	Exon 17	AGTCTAGGTGTAATACTGAGGAGG	51591407	ATCAAGTGCTCAACTGGAAACT	51590986	
	Exon 18	GTGAAGAACTTTGAGGAGAAGAC	51580461	CATCCTGTGCTGTCACTTGA	51590026	
	Exon 19	ATTTGTCCAGTGACGCTGTC	51520048	CGAATCTATTTTTTTTTTGTCAC	51519715	
	Exon 20	GAGAAGTGAATTTGTTTGGAG	51478265	TTTGTTAGTGAGAATGTGAGATGG	51477926	
	Exon 21	TATCTTCCCATTTTTCTCTTCTC	51383644	TGCCAGTGTTCTAAAAAGTATAAA	51383225	
	Exon 22	GTATAAAAACAGGAAAATGCTGA	51367510	ATAAGGGTGACAGGACAGAAAG	51367125	
	Exon 23	GTTGTCTCATAGTGTGGCTCCTC	51364206	TATTTGTTTTAATTTGGAAAGAG	51363821	

[a] Nucleotide position of the 5' end of each primer on NT_032977.7

Table 1 Primer sequences for human *DPYD*

Amplified and sequenced region			Forward primer		Reverse primer		PCR product (bp)
			Sequences (5' to 3')	Position[a]	Sequences (5' to 3')	Position[a]	
First PCR	5'-UTR to exon 1		GTTCTGGAAGGTAATCTGATGG	52207178	ACGACATACAGGAGGTGAAG	52205443	1,736
	Mix 1	Exon 2	CTACTTGGGAGACTAAGGTG	52168526	GTATCATTGTGTCATTAGGC	52167832	695
		Exon 3	TCCCTTCATCTTAGTCAATG	52113605	CTGAGGCTTAACATTTATGC	52112876	730
		Exon 4	TCTGAGAGGAGGGACAGTTA	52025660	AATCACAACTTGGAAGTGCT	52025165	496
		Exon 5	AAATGGAGGATAACCTGAGT	52007046	TAATAACCTGCTGGGATTGC	52006234	813
		Exon 6	AGAGGAGAGGCACTTAATGT	51984772	TGCTTCAAGCCAACTGCAAA	51984115	658
	Mix 2	Exon 7	CTCAAATAATAGTGCCATAGG	51977410	CAGTAGACAGACAAATGCCC	51976498	913
		Exon 8	CACATCGTGCTTTGAACATA	51964415	CCAACTCCATCCTTTATGAT	51963667	749
		Exons 9 and 10	TGAGGCAAGAATATAACCTG	51880431	TCCGTATGTGTCTTATTACC	51877795	2,637
		Exon 11	AGAAATACCTTATGATGCCG	51859160	GCCTTTTGAATCAAGATTGC	51858562	599
		Exon 16	CTCCCTATGCTTCAGTTCAC	51658925	TGCCGTGCCCCATTTACTAC	51658114	812
	Mix 3	Exon 12	CCGCTCTGAAACATTGACCA	51834944	CTGGGATTATAGGCATTAGG	51834279	666
		Exon 13	GCCCATATCTCTGAGCACTA	51801258	ATCTTTGTTGCTTCCTAGAC	51800450	809
		Exon 14	CCTTCACTGATTTACATCGG	51735640	CCAGCCACATACAGTGAAAA	51734704	937
		Exon 15	AGCCAGTAAAATCCTCTCTA	51667711	TATGGAAAACCTGCTGACTA	51666815	897
		Exon 23	TGGAAAGACCCGAACTCTGC	51364409	AGCGAAGGGGATTTTACTTA	51363336	1,074
	Mix 4	Exons 17 and 18	TTCTAAAGGCTCTGTTGAGG	51591491	TGGCAAAAGAACTGAGAGAC	51589933	1,559
		Exon 19	CGTGGATTCAAGCAGTTTTC	51520500	AGACAGTGGGTTCGTAAGCC	51519586	915
		Exon 20	CTGTGACACCATTACCATTG	51478435	TGCCAGTCATCACCACAGTA	51477733	703
		Exon 21	GAACCTGATACCGAGAAGAC	51383758	AAATGTCCAGGCTTTCCAGA	51382987	772
		Exon 22	GCCATAACAACTCACACGGG	51367740	TTGGCAGAAGGAATCATAGC	51366885	856
Second PCR		5'-UTR to exon 1	TGTGGATGTTTTTGCTCGC	52206503	AGTAAACAGGTCCCGACGC	52205586	918
		Exon 2	GTGAACTGAGATTGTACCACTGC	52168471	CATATCCCTTATCAAAAATGCTT	52167924	548
		Exon 3	GAATGCTACCCAATTAAAGTGG	52113285	TTCAAAACCAAATACAGCCTC	52112899	387
		Exon 4	TGCCAAAGATGAAACACAGA	52025601	ACCCACAGATAATAGAGAACAAGA	52025273	329
		Exon 5	TGATGGTTCCTGATAGTAGTATTG	52006775	TGTCACACTAAAAATGTTGGG	52006348	428
		Exon 6	AAGGAAAGACTGAAAGTTAGCC	51984688	GAGCCTGAAGTTCCTATATGAT	51984201	488
		Exon 7	TTCTACTGTATCTTCACTCCACG	51976953	GCTTCTGCCTGATGTAGC	51976541	413
		Exon 8	GGCTGACTTTTCATTCTTTTT	51964221	CATCTTGCCGAAATCTCTCC	51963831	391
		Exon 9	TGTGATTTACGATGTGTACTTGG	51880335	GCAAGGTTGGGTGTGAGAG	51879895	441
		Exon 10	AAAATGGGAATAAAACTGTCTT	51878507	TCAGGATATGGAAGACTTAGCAC	51877859	649
		Exon 11	ACTGGTAACTGAAACTCAG	51859069	CAATTCCCTGAAAGCTAG	51858628	442
		Exon 12	TCAGTGCCCTTCAAATGTGT	51834881	ACCAAATAGAAATGCTCTTATAGA	51834414	468
		Exon 13	TCGGATGCTGTGTTGAAGTG	51800982	TGTGTAATGATAGGTCGTGTC	51800543	440

Introduction

Dihydropyrimidine dehydrogenase (DPD) is an inactivating and rate-limiting enzyme for 5-fluorouracil (5-FU), which is used in various therapeutic regimens for gastrointestinal, breast and head/neck cancers (Grem 1996). While the antitumor effect of 5-FU is exerted via anabolic pathways responsible for its intracellular conversion into anti-proliferative nucleotides, DPD affects 5-FU availability by rapidly degrading it to 5, 6-dihydrofluorouracil (DHFU) (Heggie et al. 1987). The importance of DPD in 5-FU metabolism was also highlighted by a lethal drug interaction between 5-FU and the antiviral agent sorivudine. Due to inhibition of DPD by a sorivudine metabolite, severe systemic exposure to 5-FU caused several acute deaths in Japan (Nishiyama et al. 2000).

5-FU catabolism occurs in various tissues, including tumors, but is highest in the liver (Naguib et al. 1985; Lu et al. 1993). Wide variations in DPD activity (8- to 21-fold) were shown in Caucasians, and 3–5% of Caucasians had reduced DPD activity (Etienne et al. 1994; Lu et al. 1998). This variability, which is partially attributed to genetic defects of the DPD gene (*DPYD*), leads to differential responses of cancer patients, resistance to or increased toxicity of 5-FU (van Kuilenburg 2004). Complete DPD deficiency is also associated with the inherited metabolic disorder, thymine-uraciluria, which is characterized by neurological problems in pediatric patients (Bakkeren et al. 1984).

To date, at least 30 variant *DPYD* alleles have been published, with or without deleterious impact upon DPD activity (Gross et al. 2003; Ogura et al. 2005; Seck et al. 2005; van Kuilenburg 2004; Zhu et al. 2004). Of these variations, a splice site polymorphism, IVS14 + 1G>A, which causes skipping of exon 14, is occasionally detected in North Europeans with allele frequencies of 0.01–0.02 (van Kuilenburg 2004). Detection of IVS14 + 1G>A in patients suffering from 5-FU-associated grade 3 or 4 toxicity revealed that 24–28% of them were heterozygous or homozygous for this single nucleotide polymorphism (SNP) (van Kuilenburg 2004). However, this SNP has not been reported in Japanese and African-Americans. Recently, Ogura et al. (2005) have shown that a Japanese population exhibits a large degree of interindividual variations in DPD activity of peripheral blood mononuclear cells. They also identified a novel variation, 1097G>C (Gly366Ala), in a healthy volunteer with the lowest DPD activity and demonstrated that the 366Ala variant has reduced activity towards 5-FU in vitro. At present, however, information on variant alleles with clinical relevance in Japanese is limited and cannot fully explain polymorphic DPD activity.

In this study, we searched for genetic variations in *DPYD* by sequencing 5′ regulatory regions, all exons and surrounding introns from 341 Japanese subjects. Fifty-five variations including nine novel nonsynonymous ones were identified. Then, linkage disequilibrium (LD) and haplotype analyses were performed to clarify the *DPYD* haplotype structures in Japanese.

Materials and methods

Human DNA samples

Three hundred and forty-one Japanese subjects in this study included 263 cancer patients and 78 healthy volunteers. All 263 patients were administered 5-FU or tegafur for treatment of various cancers (mainly stomach and colon) at the National Cancer Center, and blood samples were collected prior to the fluoropyrimidine chemotherapy. The healthy volunteers were recruited at the Tokyo Women's Medical University. DNA was extracted from the blood of cancer patients and Epstein-Barr virus-transformed lymphoblastoid cells derived from healthy volunteers. Written informed consent was obtained from all participating subjects. The ethical review boards of the National Cancer Center, the Tokyo Women's Medical University and the National Institute of Health Sciences approved this study.

PCR conditions for DNA sequencing

To amplify 22 exons (exons 2–23) of *DPYD*, multiplex PCRs were performed by using four sets of mixed primers (mix 1 to mix 4 of "first PCR" in Table 1). Namely, five exonic fragments were simultaneously amplified from 50 ng of genomic DNA using 0.625 units of Ex-Taq (Takara Bio. Inc., Shiga, Japan) with 0.20 μM primers. Because of the high GC content in exon 1 of *DPYD*, this region was separately amplified from 50 ng of genomic DNA with 2.5 units of LA-Taq and 0.2 μM primers (listed in Table 1) in GC buffer I (Takara Bio. Inc.). The first PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min; and then a final extension for 7 min at 72°C. Next, each exon was amplified separately from the first PCR products by nested PCR (2nd PCR) using the primer sets (0.2 μM) listed in "second PCR" of Table 1. The second PCR conditions were the same as those of the first PCR, and LA-Taq (2.5 units) for exon 1 and Ex-Taq (0.625 units) for exons 2–23 were used. All PCR primers were designed in the flanking intronic sites to analyze the exon-intron splice junctions. The PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH) and sequenced directly on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems,

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ORIGINAL ARTICLE

Genetic variations and haplotype structures of the *DPYD* gene encoding dihydropyrimidine dehydrogenase in Japanese and their ethnic differences

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Abstract Dihydropyrimidine dehydrogenase (DPD) is an inactivating and rate-limiting enzyme for 5-fluorouracil (5-FU), and its deficiency is associated with a risk for developing a severe or fatal toxicity to 5-FU. In this study, to search for genetic variations of *DPYD* encoding DPD in Japanese, the putative promoter region, all exons, and flanking introns of *DPYD* were sequenced from 341 subjects including cancer patients treated with 5-FU. Fifty-five genetic variations, including 38 novel ones, were found and consisted of 4 in the 5′-flanking region, 21 (5 synonymous and 16 nonsynonymous) in the coding exons, and 30 in the introns. Nine novel nonsynonymous SNPs, 29C>A (Ala10Glu), 325T>A (Tyr109Asn), 451A>G (Asn151Asp), 733A>T (Ile245Phe), 793G>A (Glu265Lys), 1543G>A (Val515Ile), 1572T>G (Phe524Leu), 1666A>C (Ser556-Arg), and 2678A>G (Asn893Ser), were found at allele frequencies between 0.15 and 0.88%. Two known nonsynonymous variations reported only in Japanese, 1003G>T (**11*, Val335Leu) and 2303C>A (Thr768Lys), were found at allele frequencies of 0.15 and 2.8%, respectively. SNP and haplotype distributions in Japanese were quite different from those reported previously in Caucasians. This study provides fundamental information for pharmacogenetic studies for evaluating the efficacy and toxicity of 5-FU in Japanese and probably East Asians.

Keywords *DPYD* · SNP · Haplotype · Japanese · 5-fluorouracil

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Relationship between Expression of Vascular Endothelial Growth Factor in Tumor Tissue from Gastric Cancers and Chemotherapy Effects: Comparison between S-1 alone and the Combination of S-1 plus CDDP

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Relationship between Expression of Vascular Endothelial Growth Factor in Tumor Tissue from Gastric Cancers and Chemotherapy Effects: Comparison between S-1 alone and the Combination of S-1 plus CDDP

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Background: We have reported that vascular endothelial growth factor (VEGF) expression in gastric cancers might be a selective marker between 5-fluorouracil (5-FU) and a combination of 5-FU plus cisplatin (CDDP). In this study, the relationship between VEGF expression and effects of S-1 with and without CDDP is investigated.
Methods: The subjects were 44 patients treated with S-1 (40 mg/m^2, twice daily, days 1–28, repeated every 6 weeks) and 24 patients treated with S-1 plus CDDP (S-1 40 mg/m^2, twice daily, days 1–21, CDDP, 60 or 70 mg/m^2, day 8, repeated every 5 weeks). VEGF expression in pretreatment endoscopic biopsy samples was assessed immunohistochemically.
Results: Median survival times (MST) of the patients treated with S-1 and S-1 plus CDDP were 344 and 388 days. Among evaluable patients, the response rates of patients with VEGF (+) and (−) tumors to S-1 were 40% (6/15) and 54% (13/24), and to S-1 plus CDDP, 79% (15/19) and 80% (4/5). While the survival of patients with VEGF (−) tumors was slightly longer than those with VEGF (+) tumors in the S-1 group (MST, 425 versus 308 days, $P = 0.42$), patients with VEGF (+) tumors survived remarkably longer than those with VEGF (−) tumors in the S-1 plus CDDP group (MST, 570 versus 333 days, $P = 0.19$).
Conclusion: Similarly to our previous study, it is suggested that the effects of adding CDDP to S-1 might be more remarkable in gastric cancer patients with VEGF (+) tumors than in those with VEGF (−) tumors. These results should be confirmed in a large phase III study.

Key words: vascular endothelial growth factor – S-1 – gastric cancer

INTRODUCTION

The prognosis of patients with unresectable and recurrent gastric cancer is still poor. 5-fluorouracil (5-FU)-based chemotherapy has been widely used for advanced gastric cancer, showing a survival benefit compared with best supportive care (1). In randomized phase III trials, the survival benefit of additional cisplatin (CDDP) to 5-FU has not been clarified (2–4). In the phase III study of the Gastrointestinal Oncology Study Group in the Japan Clinical Oncology Group, although combination therapy of 5-FU plus cisplatin (FP) showed a higher response rate and longer time to progression than continuous infusion of 5-FU (5-FUci), the survival with these two regimens were identical and 5-FUci was less toxic than FP (4).

Recently, many chemotherapy regimens including new agents have been developed that show high response rates for advanced gastric cancer (5–8). S-1 is a new oral fluoropyrimidine, consisting of tegafur (FT), 5-chloro-2, 4-dihydropyrimidine (CDHP) and potassium oxonate. The two phase II studies of S-1 for advanced gastric cancer showed a response rate of 45% in total, with low incidences of severe toxicities (9,10). A combination of S-1 plus CDDP showed a very high response rate of 74% in a phase I/II study

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(11). In Japan, S-1-based regimens have been widely used in clinical practice for gastric cancer and a randomized phase III study comparing S-1 with S-1 plus CDDP is underway.

It has been generally considered that additional CDDP may bring a benefit to some patients with tumors sensitive to CDDP, whereas it may deteriorate the quality of life in patients with tumors refractory to it. Thus, it is necessary to differentiate patients to be treated with or without additional CDDP.

Progress in basic research has revealed many factors and mechanisms implicated in sensitivity and resistance to chemotherapy. In our first report of the phase II study of FP, patients with positive expression of vascular endothelial growth factor (VEGF) in their primary tumors showed a significantly higher response rate than those negative for VEGF (12). In our second report, patients with VEGF (+) tumors showed a higher response rate than those with VEGF (−) tumors after treatment with a combination of irinotecan plus CDDP (13). Moreover, in our third report, patients with VEGF (+) tumors showed a shorter survival than those with VEGF (−) tumors after treatment with 5-FUci, while there was no difference in survival between patients with VEGF (+) and (−) tumors after treatment with FP (14). These results suggest that patients with VEGF (+) tumors might receive a greater benefit from chemotherapy containing CDDP than those with VEGF (−) tumors. However, these results should be recapitulated in other cohorts.

In this study, we investigate the relationship between the expression of VEGF and chemotherapy effects of S-1 alone and S-1 plus CDDP in advanced gastric cancer patients to confirm our previous results that VEGF might be a selective marker for the addition of CDDP.

PATIENTS AND METHODS

Patient Population

The subjects of this study consisted of two groups. One was 24 of 25 patients enrolled in the phase I/II study of S-1 combined with CDDP (11). The other group was 44 consecutive patients recruited from 99 patients registered to the post-marketing survey of S-1 (15) from the National Cancer Center Hospital East between April in 1998 and March in 2000. The recruitment criteria for the S-1 group was the same as the eligibility criteria of the phase I/II study of S-1 plus CDDP (11): histologically proven gastric adenocarcinoma; age, 20–74 years; performance status 0–2 on the ECOG scale; no prior chemotherapy; and adequate bone marrow, liver and renal function. Most importantly, endoscopic biopsy samples taken from primary tumors before chemotherapy were available.

Treatment Schedule

The treatment schedule with S-1 was oral administration at a dose that did not exceed 40 mg/m^2 based on the patient's body surface area (BSA): BSA < 1.25 m^2, 40 mg; 1.25 m^2 $\leq$ BSA < 1.5 m^2, 50 mg; and BSA ≥ 1.5 m^2, 60 mg. This was administered twice daily for 28 consecutive days, followed by 2 weeks rest. In the S-1 plus CDDP group, the same dose of S-1 was administered for 21 consecutive days and CDDP at a dose of 60 (18 patients) or 70 (six patients) mg/m^2 was given intravenously with adequate hydration and repeated every 5 weeks. Both treatments were repeated until disease progression, unacceptable toxicity, or patient refusal.

Evaluation of Antitumor Effects

Tumor responses were evaluated according to the classification of the Japanese Research Society for Gastric Cancer (16), using endoscopy, X-ray imaging or CT scanning. The survival time was calculated from the initial date of the therapy to the date of death from any cause or last confirmation of survival.

Immunohistochemistry

Biopsy samples were immunostained as described in our previous studies (12–14). All immunohistochemical examinations were performed on tissue sections from formalin-fixed and paraffin-embedded biopsy materials from primary tumors. Serial 3-μm thick slices were cut, deparaffinized in xylene, dehydrated with graded ethanol and then immersed in methanol with 0.3% H_2O_2 for 20 min to inhibit endogenous peroxidase activity. The sections were treated with 0.05% pepsin in 0.01 N HCl for 20 min at room temperature. After blocking with 10% normal swine serum in phosphate-buffered saline (PBS; blocking buffer) for 60 min, all sections were incubated overnight at room temperature with the primary antibodies (polyclonal; Santa Cruz Biochemistry, CA, USA) diluted in blocking buffer to 1:500. The sections were washed with PBS and then incubated for 1 h with biotinylated secondary antibody diluted to 1:200. After washing with PBS, the sections were incubated with ABC reagent (Vector Laboratories, CA, USA), and the color reaction was developed in 2% 3-3′-diaminobenzidine and 0.3% H_2O_2 in Tris buffer. The sections were then counterstained with hematoxylin or methyl green.

All immunostained specimens were assessed by one investigator (N.B.) who was blinded to all clinical information. The VEGF staining (Fig. 1) was graded as (++) when the intensity of staining in cancer cells was stronger than that in stromal cells, as (+) when they were equal and as (−) when weaker. Patients were defined as positive when more than 20% of all cancer cells in each section were (++) or (+).

Statistical Analysis

Survival curves were calculated by the Kaplan–Meier method and compared with the log-rank test. Patient characteristics and response rates were compared with a χ^2 test or Fisher's exact test.

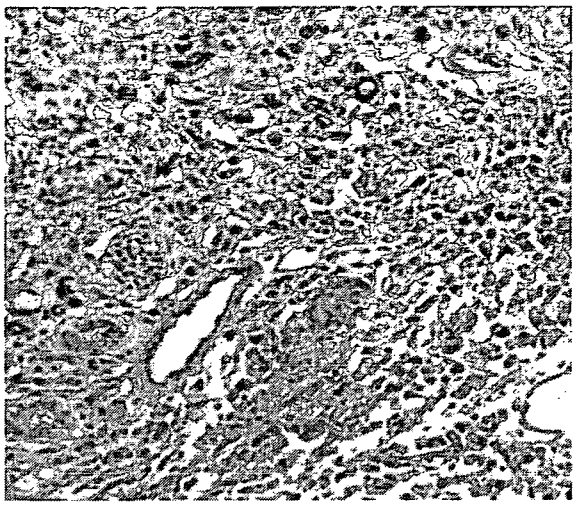

Figure 1. Expression of VEGF detected by immunohistochemistry. VEGF, vascular endothelial growth factor.

RESULTS

Subjects

Table 1 shows the clinicopathological features of the subjects. The median age was around 60 years in both treatment groups. Forty-three of the 44 patients in the S-1 group and all 24 patients in the S-1 plus CDDP group had a good performance status of one or less. Histologically, 31 patients (70%) in the S-1 group and 15 (63%) in the S-1 plus CDDP group had diffuse type adenocarcinoma. Twenty-eight patients (64%) in the S-1 group and 20 (83%) in the S-1 plus CDDP group had one or less metastatic sites.

Expression of VEGF and Clinicopathological Features

Fifteen of the 44 patients (34%) in the S-1 group had VEGF (+) tumors, while the tumor VEGF positive rate was 21% (5/24) in the S-1 plus CDDP group. Table 2 shows the clinicopathological features of patients with VEGF (−) and (+) tumors in the S-1 group and the S-1 plus CDDP group. In the S-1 group, patients with VEGF (+) tumors were significantly younger than those with VEGF (−) tumors, and other factors related to prognosis such as performance status, tumor extent and number of metastatic sites were slightly better in patients with VEGF (+) tumors than in those with VEGF (−) tumors. In the S-1 plus CDDP group, patient characteristics, except age and histological type, were well balanced between VEGF (−) and (+) tumors.

Table 1. Patient characteristics

	S-1 ($n = 44$)	S-1 + CDDP ($n = 24$)
Age (median)	59 (range 28–78)	60 (range 31–72)
Gender (M/F)	22/22 (50/50)	20/4 (83/17)
PS (0/1/2)	31/12/1 (71/27/2)	19/5/0 (79/21/0)
Histological type (intestinal/diffuse)	13/31 (30/70)	9/15 (37/63)
Resection of primary tumor (−/+)	27/17 (61/39)	19/5 (79/21)
Tumor extent (locally advanced, metastatic)	28/16 (64/36)	16/8 (67/33)
No. of metastatic sites (0/1/2/3 or more)	1/27/14/2 (2/61/32/5)	1/19/3/1 (4/79/13/4)

CDDP, cisplatin; PS, performance status.

Expression of VEGF and Response to Chemotherapy

Among the 39 patients (89%) with evaluable lesions in the S-1 group, the response rate was 49% (19/39); the response rate of all 24 patients in the S-1 plus CDDP was 79% (Table 3). In the S-1 group, the response rate of the 24 patients with VEGF (−) tumors (54%) was slightly higher than that of the 15 patients with VEGF (+) tumors (40%) ($P = 0.39$). In the S-1 plus CDDP group, the response rates of the patients with VEGF (+) and (−) were very similar.

Expression of VEGF and Survival

The median survival times (MST) for the S-1 and S-1 plus CDDP groups were 344 and 388 days, respectively (Fig. 2). Figure 3 shows the survival curves of the patients with VEGF (+) and (−) tumors in the S-1 group (A) and the S-1 plus CDDP group (B). In the S-1 group, the MST of the 29 patients with VEGF (−) tumors was 425 days and that of the 15 patients with VEGF (+) tumors was 308 days ($P = 0.42$). In the S-1 plus CDDP group, the survival of the five patients with VEGF (+) tumors was remarkably long (MST, 570 days), while the MST of the 19 patients with VEGF (−) tumors was 333 days ($P = 0.19$). In the 48 patients with VEGF (−) tumors, the 29 treated with S-1 survived relatively longer than the 19 patients treated with S-1 plus CDDP (MST, 425 days versus 333 days, $P = 0.23$). For the 20 patients with VEGF (+) tumors, five patients treated with S-1 plus CDDP showed a longer survival than the 15 patients treated with S-1 (MST, 570 days versus 308 days, $P = 0.24$).

DISCUSSION

In this study, patients were recruited from two sources; one a registry of a post-marketing survey of S-1 (15), the other a phase I/II study of S-1 plus CDDP (11). The response rate and MST of patients treated with S-1 were 49% (19/39 in evaluable patients) and 344 days, and for those treated with S-1 plus CDDP, 79% (19/24) and 384 days, respectively. Two phase II studies of S-1 showed a response rate of 45% in total, with a MST of 9 months. After the above phase I/II study of S-1 plus CDDP, subsequent

Table 2. Expression of VEGF and clinicopathological features

	S-1			S-1 + CDDP		
	VEGF (−) ($n = 29$)	VEGF (+) ($n = 15$)	P	VEGF (−) ($n = 19$)	VEGF (+) ($n = 5$)	P
Age (median)	63 (39–78)	52 (28–66)	<0.01	59 (31–72)	63 (49–72)	0.41
Gender (M/F)	13/16	9/6	0.34	16/3	4/1	>0.99
PS (0/1/2)	19/9/1	12/3/0	0.53	15/4/0	4/1/0	>0.99
Histological type (intestinal/diffuse)	9/20	4/11	>0.99	6/13	3/2	0.33
Resection of primary tumor (−/+)	18/11	9/6	>0.99	15/4	4/1	>0.99
Tumor extent (locally advanced, metastatic)	17/12	11/4	0.51	13/6	3/2	>0.99
No. of metastatic sites (0/1/2/3 or more)	1/18/8/2	0/9/6/0	0.56	1/15/2/1	0/4/1/0	0.85

VEGF, vascular endothelial growth factor.

studies of similar combination chemotherapy also reported high response rates and long MSTs (17,18). It is considered that the subjects in this study could reflect the general outcomes of gastric cancer patients treated with S-1 alone and S-1 plus CDDP.

VEGF promotes angiogenesis and the permeability of blood vessels and is associated with microvessel counts and metastasis (19–21). It has been reported that VEGF is a marker of poor prognosis after surgical resection in various kinds of malignancy, including gastric cancer (22–30). It seems that cancers producing VEGF may have a more malignant potential than those not producing VEGF. In our previous report, after treatment with 5-FUci, patients with VEGF (−) tumors showed a slightly higher response rate and significantly longer survival than those with VEGF (+) tumors (14). In this study, after treatment with S-1, the patients with VEGF (−) tumors showed a slightly higher response rate and relatively longer survival than those with VEGF (+) tumors. Comparing the characteristics between patients with VEGF (−) and (+) tumors in the S-1 group, there were more patients with favorable prognostic factors such as good performance status and local advance disease in the VEGF (+) subgroup than in the VEGF (−) subgroup. It is speculated that the difference in survival between patients with VEGF (−) and (+) tumors might be more prominent if the patient background had been well balanced. Thus, it seems that the relationship between VEGF status and chemotherapy effects such as response and survival might be common between 5-FU alone and S-1 alone.

Table 3. VEGF expression status and response

Treatment	VEGF	CR + PR	NC + PD	RR (%)	P
S-1	(−)	13	11	54	0.39
	(+)	6	9	40	
	Total	19	20	49	
S-1 + CDDP	(−)	15	4	79	>0.99
	(+)	4	1	80	
	Total	19	5	79	

CR, complete response; PR, partial response; NC, no change; PD, progressive disease; RR, response rate.

It has been reported that the response rates in the CDDP-containing regimen of the patients with VEGF (+) tumors were higher than in those with VEGF (−) tumors (12,31). In our first report on the phase II study of FP, the response rate in patients with VEGF (+) tumors was significantly higher than in those with VEGF (−) tumors, while there was no difference in survival between patients with VEGF (+) and (−) tumors (12). Then again, patients with VEGF (−) tumors survived longer than those with VEGF (+) tumors after treatment with 5-FUci (14). In this study, because the number of patients treated with S-1 plus CDDP was small, the difference was not statistically significant. The patients with VEGF (+) tumors survived remarkably longer than those with VEGF (−) tumors after treatment with S-1 plus CDDP, while the survival of the patients with VEGF (−) tumor was slightly longer than in those with VEGF (+) tumors after treatment with S-1 alone. Considering the results of our previous study (5-FU and FP) and this study (S-1 and S-1 plus CDDP), the relationship between VEGF status and the effects of CDDP additional to 5-FU-based drugs seemed to be similar. Moreover, while the patients with VEGF (−) tumors showed a slightly higher response rate than those with VEGF (+) tumors, those of S-1 plus CDDP were similar between VEGF (−) and (+). The difference in response rate between S-1 alone and S-1 plus CDDP of VEGF (+) subgroup was larger than VEGF (−) subgroup. It is speculated that there might be some unknown mechanisms related to sensitivity to CDDP in gastric cancers producing VEGF and that the addition of CDDP might overcome the malignant potential of VEGF (+) tumor patients.

In conclusion, the relationship between VEGF status and chemotherapeutic effects that had been observed in 5-FU-based chemotherapy with and without additional

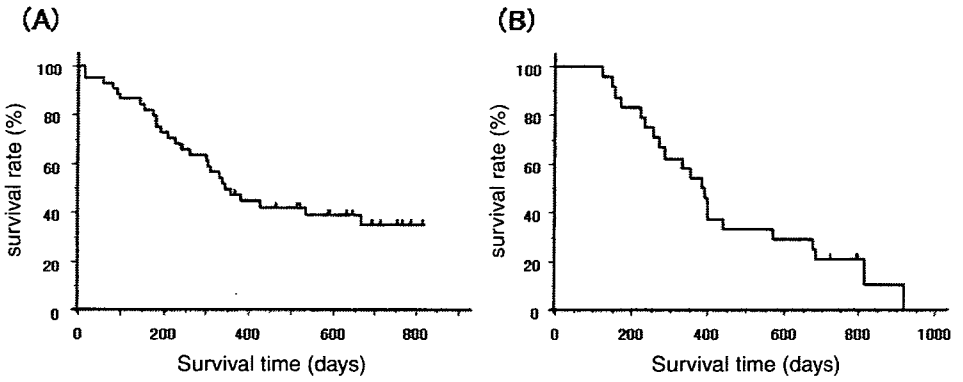

Figure 2. Overall survival of S-1 (A) and S-1 plus CDDP (B) patients. CDDP, cisplatin.

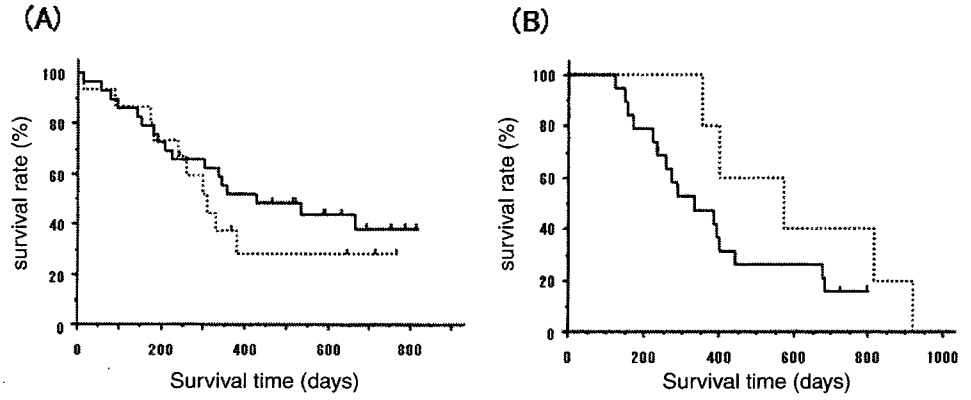

Figure 3. Survival and VEGF expression status in S-1 (A) and S-1 plus CDDP (B) groups. Solid line, VEGF (−) and dotted line, VEGF (+).

CDDP may be reproduced using S-1 and S-1 plus CDDP. It is suggested that the effects of adding CDDP to S-1 might be more remarkable in gastric cancer patients with VEGF (+) tumors than in those with VEGF (−) tumors. These results should be confirmed in a large phase III study.

Acknowledgment

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Conflict of interest statement

None declared.

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Haplotypes and a Novel Defective Allele of *CES2* Found in a Japanese Population

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ABSTRACT:

Human carboxylesterase 2 (hCE-2) is a member of the serine esterase superfamily and is responsible for hydrolysis of a wide variety of xenobiotic and endogenous esters. hCE-2 also activates an anticancer drug, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, CPT-11), into its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38). In this study, a comprehensive haplotype analysis of the *CES2* gene, which encodes hCE-2, in a Japanese population was conducted. Using 21 single nucleotide polymorphisms (SNPs), including 4 nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$, **2*), 424G>A ($Val^{142}Met$, **3*), 1A>T ($Met^{1}Leu$, **5*), and 617G>A ($Arg^{206}His$, **6*), and a SNP at the splice acceptor site of intron 8 (IVS8-2A>G, **4*), 20 haplotypes were identified in 262 Japanese subjects. In 176 Japanese cancer patients who received irinotecan, associations of *CES2* haplotypes and changes in a pharmacokinetic parameter, (SN-38 + SN-38G)/CPT-11 area under the plasma concentration curve (AUC) ratio, were analyzed. No significant association was found among the major haplotypes of the **1* group lacking nonsynonymous or defective SNPs. However, patients with nonsynonymous SNPs, 100C>T ($Arg^{34}Trp$) or 1A>T ($Met^{1}Leu$), showed substantially reduced AUC ratios. In vitro functional characterization of the SNPs was conducted and showed that the 1A>T SNP affected translational but not transcriptional efficiency. These findings are useful for further pharmacogenetic studies on CES2-activated prodrugs.

Human carboxylesterases are members of the serine esterase superfamily and are responsible for hydrolysis of a wide variety of xenobiotic and endogenous esters. They metabolize esters, thioesters, carbamates, and amides to yield soluble acids and alcohols or amines (Satoh and Hosokawa, 1998; Satoh et al., 2002). In the human liver, two major isoforms of carboxylesterase, hCE-1 and hCE-2, have been identified (Shibata et al., 1993; Schwer et al., 1997). hCE-2 is a 60-kDa monomeric enzyme with a pI value of approximately 4.9 and shares 48% amino acid sequence identity with hCE-1 (Pindel et al., 1997; Schwer et al., 1997; Takai et al., 1997). The *CES2* gene, which encodes hCE-2, is located on chromosome 16q22.1 and consists of 12 exons. Distribution of hCE-2 is relatively limited to several tissues, such as the small intestine, colon, heart, kidney, and liver, whereas hCE-1 is ubiquitously expressed (Satoh et al., 2002; Xie et al., 2002).

Although both hCE-1 and hCE-2 show broad substrate specificities, hCE-2 is relatively specific for heroin, cocaine (benzoyl ester), 6-acetylmorphine, procaine, and oxybutynin (Pindel et al., 1997; Takai et al., 1997; Satoh et al., 2002). In addition, hCE-2 is reported to play a major role in the metabolic activation of the antitumor drug irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin; CPT-11). Irinotecan is a water-soluble derivative of the plant alkaloid camptothecin and is widely used for treatment of several types of cancer. Irinotecan is converted to 7-ethyl-10-hydroxy-

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ABBREVIATIONS: hCE-1, human carboxylesterase 1; hCE-2, human carboxylesterase 2 (EC 3.1.1.1); irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, CPT-11; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38G, SN-38 glucuronide; SNP, single nucleotide polymorphisms; PCR, polymerase chain reaction; LD, linkage disequilibrium; 5-FU, 5-fluorouracil; MMC, mitomycin C; AUC, area under plasma concentration curve; RT, reverse transcriptase; UTR, untranslated region; ORF, open reading frame.