between irinotecan dosage and the AUC of SN-38 were tested, different correlations were obtained according to the number of the haplotypes (Fig. 3c). The slope of regression line for one and two haplotypes harboring *6 or *28 was 1.4-fold and 2.4-fold greater, respectively, than that for the diplotype without *6 or *28.

Associations of UGT1A1 genetic polymorphisms with toxicities

Association between genetic polymorphisms and toxicities was investigated in patients receiving irinotecan as a single agent. One patient was referred to another hospital 3 days after the first administration of irinotecan without evaluating toxicities and was lost in terms of follow-up. Therefore, association between genetic polymorphisms and toxicities was investigated in 55 patients. Six (11%) and 14 (25%) patients experienced grade 3 or greater diarrhea and neutropenia, respectively. As for the *1A9-1A7-1A1* diplotypes, a higher incidence of grade 3 or greater neutropenia was observed in *D1/B2* (*1A1*28/*6*) (100%, $n = 3$) than in *A1/A1* (11.8%, $n = 17$) ($P = 0.0088$, Fisher's exact test), indicating clinical impact of the genetic marker *1A1*6* or *28. As for the dose effect of '*6 or *28', incidences of grade 3 or 4 neutropenia were 14, 24, and 80% for 0, 1, and 2 haplotypes harboring these markers, respectively (Table 5). A significant association between '*6 or *28'and neutropenia was also observed for 62 patients who received irinotecan in combination with cisplatin (Table 5). No association, however, was observed between diarrhea and the marker '*6 or *28'.

Multivariate analysis for irinotecan toxicities

We further evaluated the effect of the genetic marker '*6 or *28' on neutropenia in multivariate analysis, and confirmed a significant correlation of '*6 or *28' with the nadir of absolute neutrophil counts (Table 6). Elevated alkaline phosphatase levels and the absolute neutrophil count at baseline were also significant.

Discussion

The association study with the *1A9-1A7-1A1* diplotypes revealed that the reduction in inactivation of SN-38, as well as neutropenia, was dependent on the Groups B and D haplotypes which corresponded to the *1A1*6* and *28 segmental haplotypes. Also, multivariate analyses clearly showed clinical significance of the genetic marker '*6 or *28' for both pharmacokinetics and toxicity of irinotecan in Japanese patients (Tables 3 and 6). *UGT1A1*6* and *28 were mutually exclusive [14] and contributed to the reduction in glucuronidation of SN-38 to the same extent. Therefore, the activity of SN-38 glucuronidation in individuals depended on the number of the haplotypes harboring *6 or *28. Although the role of *1A1*28* for irinotecan toxicity has been focused on [8–12], this study strongly suggests that *6 should be tested in addition to *28 before starting chemotherapy with irinotecan in Japanese patients.

Table 4 Multiple regression analysis toward the AUC ratio (SN-38G/SN-38)[a]

Variable	Coefficient	F-value	*P*-value	R^2	Intercept	*N*
				0.410	0.8869	176
*6 or *28	−0.189	70.2	<0.0001			
Age	0.005	8.88	0.0033			
Serum albumin level[b]	−0.136	9.92	0.0019			
Serum GOT and ALP[c]	0.070	8.88	0.0033			
Serum creatinine[d]	0.210	7.23	0.0079			

ALP, alkaline phosphatase; AUC, area under concentration curve.
[a]The values after logarithmic conversion were used as an objective variable.
[b]The absolute value (g/dl) before irinotecan treatment.
[c]Grade 1 or greater scores in both serum GOT and ALP before irinotecan treatment.
[d]Grade 1 or greater scores in serum creatinine before irinotecan treatment.

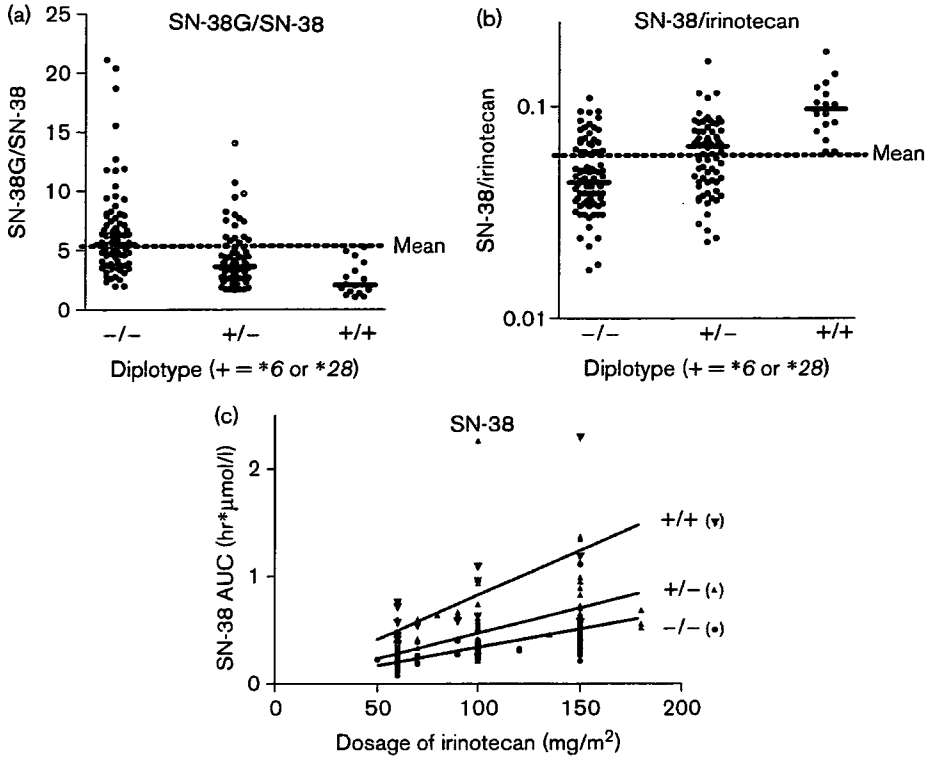
The clinical importance of *6 for neutropenia by irinotecan was also supported by a recent report in Korean patients who received irinotecan and cisplatin [31]. Although no patients with irinotecan as a single agent were homozygous for *6 in our study, clinical significance of the double heterozygote, *6/*28, was clearly demonstrated. Among patients treated with irinotecan in combination chemotherapy, the majority of patients received platinum agents in our study. A significant association of '*6 or *28' with a higher incidence of grade 3 or 4 neutropenia was also observed in patients who received irinotecan and cisplatin (Table 5). These findings further support the necessity of testing '*6 or *28' before irinotecan is given to patients.

As possible enhancement of toxicities by the *27 allele was suggested [8], we evaluated the effect of the *28c haplotype, which had an additional single-nucleotide polymorphism [*27; 686C > A(P229Q)] to the *28 allele (-40_-39insTA). In our cohort of patients, there were three *28c heterozygotes (*28c/*1) and one double heterozygote (*28b/*28c). The values of the AUC ratio were within the range of variations of the *28 group, and no additional impact of *28c was observed in relation to toxicities.

Although the decreasing trend of the AUC ratio for *1A1*60* (and combinatorial haplotype *C3*) was observed (Fig. 2), the contribution of *1A1*60* to toxicities was not clearly demonstrated in this study as reported in the Japanese retrospective study [32].

In addition to UGT1A1, recent studies have suggested possible contributions of UGT1A7, 1A9, and 1A10 to SN-38G formation [15–17]. An in-vitro study demonstrated that *1A7*3* [387T > G(N129K), 391C > A(R131K), 622T > C(W208R)] had reduced activity in terms of SN-38G formation [16]. Results of clinical studies, however, on the association between *1A7* polymorphisms and irinotecan toxicity/efficacy are inconsistent, whereas different populations with different combination therapies were used [19,20]. Furthermore, it was reported that the *UGT1A7* polymorphisms (*2 and *3), which were linked to *1A9*1*, were associated with a lowered incidence

Fig. 3



Effects of the genetic marker of *UGT1A1* '*6 or *28' on the area under concentration curve (AUC) ratios of SN-38G/SN-38 (a) and SN-38/irinotecan (b), and SN-38 by irinotecan dosage (c) in 176 Japanese cancer patients after irinotecan treatment.

Table 5 Association of *UGT1A1*6* and *28 with irinotecan toxicities

Diplotype (+ = *6 or *28)	Number of patients	Diarrhea (grade 3)	Neutropenia (grade 3 or 4)
Irinotecan monotherapy			
-/-	21	3 (14.3%)[a]	3 (14.3%)
+/-	29	2 (6.90%)	7 (24.1%)
+/+	5	1 (20.0%)	4 (80.0%)
P-value[b]		0.8500	**0.0117**
P-value[c]		0.3889	**0.0124**
With cisplatin			
-/-	35	1 (2.9%)	20 (57.1%)
+/-	20	2 (10.0%)	14 (70.0%)
+/+	7	1 (14.3%)	7 (100%)
P-value[b]		0.1747	**0.0315**
P-value[c]		0.3886	0.0863

[a]Percentage of the patient number in each diplotype is indicated in parentheses.
[b]Chi-squared test for trend.
[c]Fisher's exact test, (-/- and +/-) vs. +/+.

Table 6 Multiple regression analysis of the nadir of absolute neutrophil counts in the patients with irinotecan monotherapy

Variable	Coefficient	F-value	*P*-value	R^2	Intercept	*N*
				0.3942	643	53
Serum ALP[a]	-349.9	12.2	0.0010			
Neutrophil count before irinotecan treatment	0.2466	13.5	0.0006			
*6 or *28	-369.1	6.40	0.0146			

[a]Grade 1 or greater scores of serum ALP before irinotecan treatment.

of diarrhea in the irinotecan/capecitabine regimen, in which diarrhea was a major toxicity [20]. A highly frequent allele *1A9*22* with an insertion of T into the nine T repeats in the promoter region (-126_-118$T_9 > T_{10}$) was shown to have an enhanced promoter activity in an in-vitro reporter assay [21], whereas 1A9 protein expression levels did not change in the clinical samples [22]. Rare variations, *1A9*5* [766G > A(D256N)] and *UGT1A10*3* [605C > T(T202I)], were shown to cause reduced activity *in vitro*, but their clinical importance is still unknown [23,24]. Moreover, close linkages among *1A9*, *1A7*, and *1A1* polymorphisms were found in Caucasians and Asians in an ethnic-specific manner [20,25–28].

Our study also revealed close linkages between *1A9*22* and *1A7*1*, *1A7*3* and *1A1*6* or *28 [28]. This fact makes it difficult to draw firm conclusions about the effects of *1A7*3* and *1A9*22* themselves. It is, however, reasonable to conclude that the degree of neutropenia depends on the activity of UGT1A1, because UGT1A1 is a major UGT1A enzyme in the liver and plays a primary role for regulating plasma concentrations of SN-38.

Taken together, for practical application to individualized irinotecan therapy, genotyping of *UGT1A1*6* and *28 would be beneficial and necessary in Japanese cancer patients to avoid severe adverse reactions. The frequency

of homozygotes for '**6* or **28*' (namely, **6/*6*, **6/*28*, and **28/*28*) is approximately 10%, which is comparable to the frequency of **28* homozygotes in Caucasian populations. In our study, it may be difficult to establish definite guidelines for dose reductions of irinotecan for patients homozygous for '**6* or **28*'. Considering, however, 2.4-fold steep relationship between the dose of irinotecan and the AUC of SN-38 for patients homozygous for '**6* or **28*' compared with patients without '**6* or **28*' (Fig. 3c), the dose for patients homozygous for '**6* or **28*' should be reduced to a half of the dosage recommended for other patients. Prospective studies are necessary to confirm the validity of the recommendation for dose reduction in Japanese cancer patients homozygous for '**6 or *28*'.

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ORIGINAL ARTICLE

Analysis of *ENG* and *ACVRL1* genes in 137 HHT Italian families identifies 76 different mutations (24 novel). Comparison with other European studies

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Abstract Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder causing vascular dysplasias. About 70–80% of HHT patients carries mutations in *ENG* or *ACVRL1* genes, which code for a TGFβ receptor type III and I respectively. Molecular data on a large cohort of Italian HHT patients are presented, discussing the significance of missense and splice site mutations. Mutation analysis in *ENG* and *ACVRL1* genes was performed using single strand conformation polymorphisms (SSCP), denaturing high performance liquid chromatography (DHPLC) and subsequent direct sequencing. Overall, 101 mutations were found, with *ACVRL1* involved in 71% of cases. The highest number of mutations (28/101 subjects, 14/76 different mutations referring to both genes) was in *ACVRL1*, exon 3. Mutation analysis was then extended to a total of 356 family members, and 162 proven to carry the mutation. New polymorphisms were identified in both genes, and evidence that ENG P131L change is not a disease-causing mutation was also provided. An in silico analysis was performed in order to characterize splice-site mutations. These results were compared to other European national studies and data from Italy, France and Spain were consistent for an higher incidence of *ACVRL1* mutations.

Keywords Hereditary hemorrhagic telangiectasia · HHT · *ACVRL1* mutation · *ENG* mutation

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Introduction

Hereditary hemorrhagic telangiectasia (HHT; Mutation Database: http://137.195.14.43/cgi-bin/WebObjects/hht.woa/wa/default) is an autosomal dominant disorder causing vascular dysplasias such as mucocutaneous telangiectases and arterovenous malformations (AVMs). Telangiectases may lead to epistaxes and gastrointestinal bleeding, which may be severe enough to require transfusions. Epistaxes and telangiectases are the most frequent symptoms, present in more than 95% of the patients. AVMs are mostly observed in liver (60%), lungs (18–70%) and brain (6%), and may cause severe life-threatening complications (Lesca et al. 2007). The phenotype is highly variable, even among members of the same family, and the disease displays age-related penetrance, with increased manifestations developing over a lifetime. About 70–80% of HHT patients carry mutations in either of two genes—*ENG* (OMIM #131195) (HHT1: OMIM 187300) or *ACVRL1* (OMIM #601284) (HHT2: OMIM 600376)—which code for a TGFβ receptor type III and I respectively, although David

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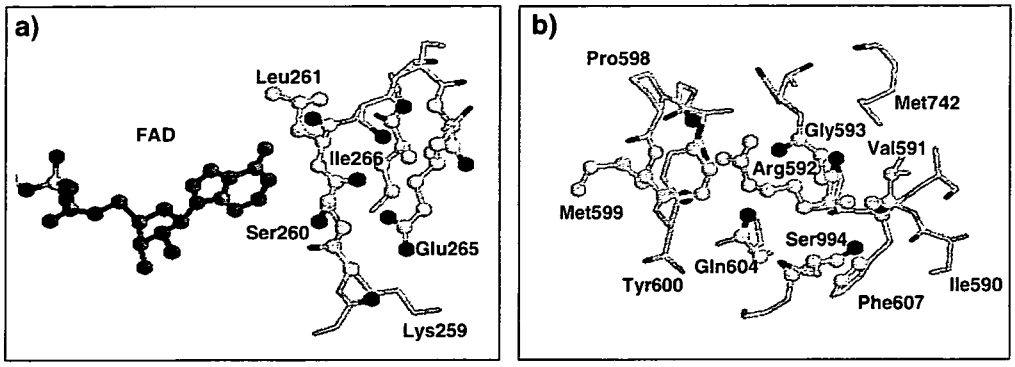

Fig. 5 Stereo view of the variation sites in pig DPD (accession code of the Protein Data Bank: 1gth). Glu265 (**a**), Arg592 (**b**) and their adjacent residues are shown as *ball-and-stick* models with oxygens in *red*, nitrogens in *blue*, carbons in *gray* and sulfur in *yellow*. The adenosine moiety of the cofactor FAD is also shown in *pink* (**a**)

Japanese and Caucasians). HapMap data on 44 unrelated Japanese subjects showed that 476 variations are polymorphic, whereas 529 are monomorphic, and the average density of polymorphic markers is 1 SNP per 1,772 bp. In contrast, our study focused on exons and surrounding introns to detect variations, and only nine variations overlapped with the HapMap data. Therefore, we could not utilize the HapMap data to further identify common subtypes of *#1* to be discriminated by many intronic HapMap SNPs in each block. However, most of the frequent SNPs are unlikely to be associated with substantially decreased DPD activity because DPD activity in the healthy Japanese population ($N = 150$) showed a unimodal Gaussian distribution (Ogura et al. 2005).

On the other hand, in 60 unrelated Caucasian subjects in the HapMap project, 617 are polymorphic, whereas 383 are monomorphic. LD profiles of these polymorphisms were compared between Caucasians and Japanese by using the program Marker (http://www.gmap.net/marker). Strong LD ($|D'| > 0.75$) clearly decays within introns 11, 12, 13, 14, 16, 18, and 20 in Japanese, whereas, similar decays are observed within introns 13, 14, 18, and 20, but are not obvious within introns 11, 12, and 16 in Caucasians (data not shown). Moreover, strong LD decays within intron 3 in Caucasians. Therefore, the LD blocks are considerably different between Japanese and Caucasians. Along with the marked differences in allele frequencies of several variations (Table 4), these results suggest that the haplotype structures in *DPYD* are quite different between the two populations.

In conclusion, we found 55 variations, including 38 novel ones, in *DPYD* from 341 Japanese subjects. Nine novel nonsynonymous SNPs were found, some of which were assumed to have impact on the structure and function of DPD. As for known variations, we obtained their accurate allele frequencies in a Japanese population of a large size and showed that variations with clinical relevance do not overlap between Caucasians and Japanese. In Japanese, 2303C>A (Thr768Lys) and 1003G>T (Val335Leu) might play important roles in 5-FU-related toxicity. Along with differences in haplotype structures between Japanese and Caucasians, these findings suggest that ethnic-specific tagging SNPs should be considered on genotyping *DPYD*. Thus, the present information would be useful for pharmacogenetic studies for evaluating the efficacy and toxicity of 5-FU in Japanese and probably in East Asians.

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Table 4 Allele frequencies of common *DPYD* SNPs in different populations

Nucleotide change (amino acid change)	Allele or tagged haplotypes	Population	Allele frequency	Number of subjects	Reference
85T>C	*9	Caucasian	0.194	157	Seck et al. 2005
(Cys29Arg)	(Block 1 #9)	French Caucasian	0.185	487	Morel et al. 2006
		Japanese	0.037	107	Yamaguchi et al. 2001
		Japanese	0.029	341	This study
		Taiwanese	0.022	300	Hsiao et al. 2004
496A>G	Block 1 #166V	Caucasian	0.080	157	Seck et al. 2005
(Met166Val)		Japanese	0.022	341	This study
IVS10-15T>C	Block 1 #166Va, #9d	Caucasian	0.127	157	Seck et al. 2005
		Japanese	0.018	341	This study
1627A>G	*5	Caucasian	0.140	157	Seck et al. 2005
(Ile543Val)	(Block 2 #5)	Caucasian	0.275	60	Ridge et al. 1998a
		Finnish	0.072	90	Wei et al. 1998
		African-American	0.227	105	Wei et al. 1998
		Japanese	0.352	50	Wei et al. 1998
		Japanese	0.283	341	This study
		Taiwanese	0.210	131	Wei et al. 1998
		Taiwanese	0.283	300	Hsiao et al. 2004
1896T>C	Block 3 #1b	Caucasian	0.035	157	Seck et al. 2005
(Phe632Phe)		Japanese	0.098	107	Yamaguchi et al. 2001
		Japanese	0.139	341	This study
		Han Chinese	0.133	45	HapMap
IVS15 + 75A>G	Block 4 #1b	Caucasian	0.166	157	Seck et al. 2005
		Japanese	0.155	341	This study
IVS16-94G>T	Block 5 #1b	Caucasian	0.415	59	HapMap
		Yorba	ND	60	HapMap
		Japanese	0.455	44	HapMap
		Japanese	0.378	341	This study
		Han Chinese	0.333	45	HapMap
2194G>A	*6	Caucasian	0.022	157	Seck et al. 2005
(Val732Ile)	(Block 5 #6)	Caucasian	0.058	60	Ridge et al. 1998a
		Finnish	0.067	90	Wei et al. 1998
		African-American	0.019	105	Wei et al. 1998
		Japanese	0.044	50	Wei et al. 1998
		Japanese	0.015	341	This study
		Taiwanese	0.014	131	Wei et al. 1998
		Taiwanese	0.012	300	Hsiao et al. 2004
IVS18-39G>A	Block 6 #1b	Caucasian	0.105	157	Seck et al. 2005
		Caucasian	0.100	60	HapMap
		Yorba	0.017	60	HapMap
		Japanese	0.044	45	HapMap
		Japanese	0.032	341	This study
		Han Chinese	0.022	45	HapMap
IVS22-69G>A	Block 6 #1f	Caucasian	0.183	60	HapMap
		Yorba	0.400	60	HapMap
		Japanese	ND	45	HapMap
		Japanese	0.003	341	This study
		Han Chinese	ND	45	HapMap

ND not detected

substitution Cys29Arg on the protein surface was unlikely to alter DPD activity. However, conflicting results were reported regarding **9* (Vreken et al. 1997, van Kuilenburg et al. 2000), **6* (van Kuilenburg et al. 2000), and Met166Val (van Kuilenburg et al. 2000; Gross et al. 2003). To interpret these inconsistencies, haplotype analysis of *DPYD* might be helpful. Especially for **9* and Met166Val in Japanese, functional involvement of –477T>G (block 1 #*9c* and #*9e*), –243G>A (block 1 #*9d*), IVS10-15T>C (block 1 #*9d* and #*166Va*) and many other HapMap SNPs linked to **9* and Met166Val (Table 3) needs clarification.

The HapMap project provides genotype data of more than 1,000 sites located mostly in the intronic regions of *DPYD* for four different populations (Nigerian, Chinese,

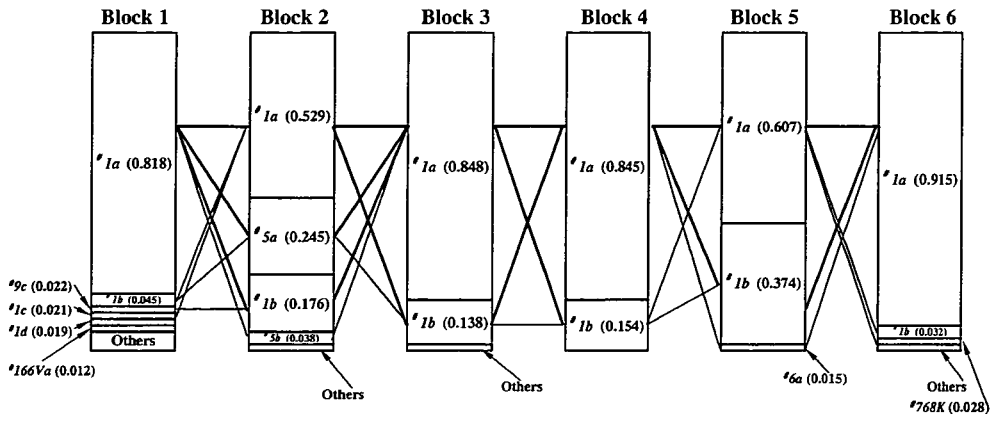

Fig. 4 The combinations of block haplotypes in Japanese. *Thick lines* represent combinations with frequencies over 10%, and *thin lines* represent combinations with frequencies of 1.0–9.9%

detected in the patient exhibiting severe mucositis during cyclophosphamide/methotrexate/5-FU chemotherapy (Gross et al. 2003). Furthermore, the adjacent Leu261 interacts via the main chain atoms with the N6, N1, and N3 atoms of adenine of FAD, and has an important role in the proper orientation of the adenine moiety in the FAD-binding pocket (Dobritzsch et al. 2001). Moreover, the carboxyl group (Glu265-Oε)might form hydrogen bonds to the main chain nitrogen of Ser260 next to Leu261. Thus, the change in polarity from negative to positive by the novel Glu265Lys substitution is likely to cause structural changes affecting proper binding of FAD.

Arg592 is located at one (IVβc) of the additional four-stranded antiparallel β sheets (IVβc-βf) inserted at the top of a typical $(\alpha/\beta)_8$ barrel fold in the FMN-binding domain IV (Dobritzsch et al. 2001). Arg592 is completely conserved among the above-mentioned six species (Mattison et al. 2002), suggesting its functional importance. Arg592 closely contacts Met599 (2.9 Å) and Gln604 (2.8 Å) in the same subunit and Ser994 (2.9 Å) in another subunit (Fig. 5B). The substitution of tryptophan for Arg592 is likely to weaken these interactions due to altered hydrophobicity and electrostatic changes. Arg592Trp was recently reported from a Korean population with an allele frequency of 0.004, although its functional significance remains to be confirmed(Cho et al. 2007).

As for known *DPYD* alleles, their distributions in several populations are becoming more evident by recent reports. For example, IVS14 + 1G>A (**2*) (van Kuilenburg 2004), 295_298delTCAT (Phe100SerfsX15, **7*) (Seck et al. 2005), 1679T>G (Ile560Ser, **13*) (Collie-Duguid et al. 2000; Morel et al. 2006) 2846A>T (Asp949Val) (Seck et al. 2005; Morel et al. 2006), all of which are associated with decreased DPD activities, are detected in Caucasians with allele frequencies of 0.01–0.02, 0.003, 0.001 and 0.006–0.008, respectively. However, none of them were detected in our Japanese samples, while 1003G>T (Val335Leu, **11*) and 2303C>A (Thr768Lys) have been found only in Japanese, indicating that variations with clinical relevance do not overlap between Caucasians and Japanese.

2303C>A (Thr768Lys), which was originally found in a Japanese female volunteer with very low DPD activity (Ogura et al. 2005), is relatively frequent in Japanese (allele frequency = 0.0279). Functional characterization in vitro revealed that 768Lys caused thermal instability of the variant protein without changing its affinity for NADPH or kinetic parameters toward 5-FU. Therefore, they might cause 5-FU-related toxicities in Japanese.

1003G>T (Val335Leu, **11*) was found in a Japanese family with decreased DPD activity by Kouwaki et al. (1998). By in vitro expression in *E. coli*, they demonstrated that the variant protein with Leu335 showed a significant loss of activity (about 17% of the wild-type protein). Dobritzsch et al. (2001) suggested from the 3D structure of pig DPD that Val335Leu, in spite of a conservative change, disturbs packing interactions in the hydrophobic core formed by IIIβ3 and IIIα3 within the Rossman-motif, thereby affecting NADPH binding. In our study, heterozygous 1003G>T (Val335Leu) was found from a patient administrated 5-FU (allele frequency = 0.0015), who also has seven other variations: IVS12–11G>A, 1896T>C (Phe632Phe), and IVS16–94G>T are heterozygous, and 1627A>G (Ile543Val), IVS13 + 39C>T, IVS14–123C>A, and IVS15 + 75A>G are homozygous, indicating that at least Val335Leu is linked to Ile543Val (**5*).

On the other hand, Caucasians and Japanese share four variations: **5* (Ile543Val), **9* (Cys29Arg), Met166Val, and **6* (Val732Ile), although their allele frequencies were different, especially for **9* (Table 4). Because they have not necessarily correlated with phenotypic changes (e.g., differences in DPD enzyme activity, 5-FU pharmacokinetics and pharmacodynamics) (Collie-Duguid et al. 2000; Johnson et al. 2002; Zhu et al. 2004; Seck et al. 2005; Ridge et al. 1998a, 1998b; Hsiao et al. 2004), all of these variations are generally accepted as common polymorphisms that result in unaltered function. Consistent with this, van Kuilenburg et al. (2002) suggested that the

Table 3 Linkages of haplotype-tagging SNPs with HapMap SNPs for *DPYD*

Haplotype-tagging SNPs in *DPYD*	dbSNP ID (NCBI)	Block haplotype in this paper	HapMap SNPs with close linkages ($r^2 > 0.8$)[a]
85T>C (Cys29Arg)	rs1801265	Block 1 #*9*	rs10747488, rs7526108, rs4421623, rs4379706, rs4523551,rs11165921, rs9661794, rs6677116, rs6604093, rs17379561, rs10747491, rs10747492, rs12062845, rs7524038, rs10875112, rs4394693, rs10875113, rs4970722, rs9727548, rs10875118, rs9662719, rs12077442, rs4394694, rs9727976, rs4246515, rs6692580
496A>G (Met166Val)	rs2297595	Block 1 #*166V*	rs2786543, rs2811215, rs2811214, rs2786544, rs2248658, rs11165897, rs2786490, rs2811203, rs2811202, rs2811200, rs2811198, rs2786503, rs2811196, rs2786505, rs2811195, rs2811194, rs12073839, rs6663670, rs7512910, rs2151563, rs2786509, rs3790387, rs3790389
1627A>G (Ile543Val)	rs1801159	Block 2 #*5*	rs1415682, rs952501, rs2811187, rs2786778, rs2786774, rs2811183, rs17116806, rs2786780, rs1801159, rs2786771, rs2297780, rs2297779, rs12729863
1896T>C (Phe632Phe)	rs7556439	Block 3 #*1b*	rs12073650
IVS16-94G>T	rs7556439	Block 5 #*1b*	rs693680, rs827500, rs499009, rs7518848, rs553388, rs507170, rs628959, rs991544, rs526645, rs1609519
IVS18-39G>A	rs12137711	Block 6 #*1b*	rs12120068, rs12116905

[a] All SNPs are in the same block

Taken together, our data demonstrated considerable differences in the haplotype distributions in blocks 1, 3 and 6 between Japanese and Caucasians.

Discussion

This study provides Japanese data on the genetic variations of *DPYD*, a gene encoding a key enzyme catalyzing degradation of the well-known anticancer drug 5-FU. Nine novel (Ala10Glu, Tyr109Asn, Asn151Asp, Ile245Phe, Glu265Lys, Val515Ile, Phe524Leu, Ser556Arg, and Asn893Ser) and seven known nonsynonymous variations (Cys29Arg, Met166Val, Val335Leu, Ile543Val, Arg592Trp, Val732Ile, and Thr768Lys) were found in our Japanese population (Table 2 and Fig. 1). The association analysis between the genotypes and 5-FU pharmacodynamics is now on-going.

Uneven distributions of coding SNPs over 23 *DPYD* exons were pointed out in the previous review by van Kuilenburg (2004). The author indicated that 81% of all reported variations were confined to exons 2–14, representing 61% of the coding sequences, and typical hotspots of variation were localized in exons 2, 6, and 13. Our Japanese data also revealed that 17 out of 21 coding variations (81%) were localized in exons 1–14, and that more than three variations were detected in exons 5, 13, and 14 (Fig. 1). Recently, Hormozian et al. (2007) have reported that the common chromosomal fragile site on 1p21.2, *FRA1E*, spans 370 kb of genomic sequence between introns 8 and 18 of *DPYD*, and that its core region with the highest fragility is located between introns 12 and 16. The instability at the core of *FRA1E* might be associated with the high mutational rates and recombinogenic nature from intron 12 to 14 of *DPYD* (Fig. 1).

To estimate potential functional consequences of the amino acid substitutions, we examined whether the positions of amino acid changes are located in highly conserved areas or potentially critical regions of the molecule (for example, substrate recognition sites or binding regions of prosthetic groups). We also considered the locations of the residues in a three-dimensional (3D) framework provided by the crystal structures of pig DPD, which have recently been determined in complexes with NADPH and substrate (5-FU) (Dobritzsch et al. 2001) or inhibitors (Dobritzsch et al. 2002). The amino acid sequences of pig and human DPD are 93% identical (Mattison et al. 2002), and the substituted residues and their neighboring residues are conserved between both enzymes. From these points of view, it is speculated that at least two substitutions (Glu265Lys and Arg592Trp) might impact the structure and function of DPD as discussed below.

Glu265 is located on the loop following to the third β sheet (IIβ3) in the FAD binding domain II (Dobritzsch et al. 2001). Glu265 is conserved among four mammalian species (human, mouse, rat, and pig), although it is replaced with aspartic acid in bovine and *Drosophila melanogaster* DPDs (Mattison et al. 2002). In the 3D structure of pig DPD (Fig. 5a), Glu265 is in close proximity to Lys259. The substitution, Lys259Glu, was

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+24 A>G	IVS10-15T>C	Number	Frequency
Amino acid change					A10E	C29R			Y109N	N151D	F158F		M166V	D213D	E245F		E265K			V335L				
Haplotype *1	*1a																						553	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	*9c					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

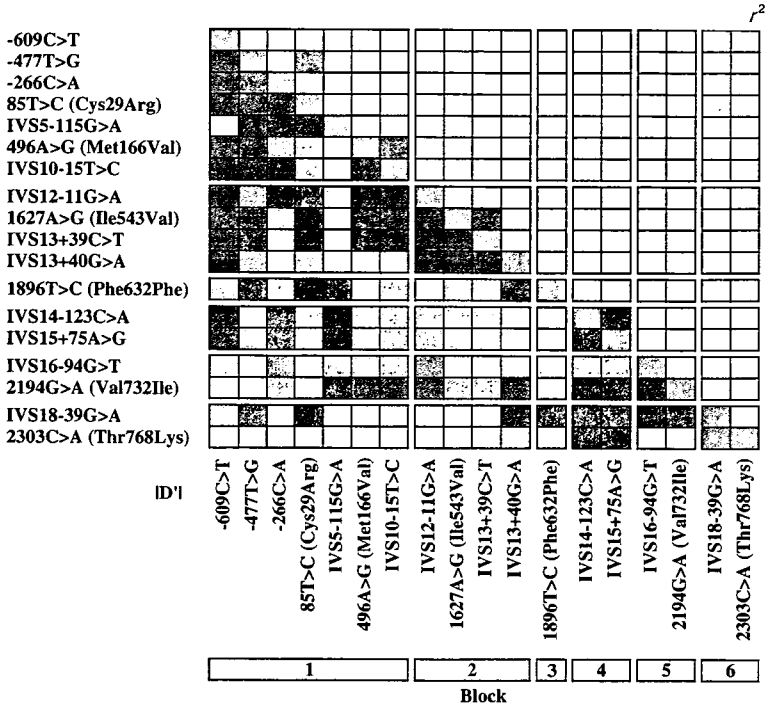

Fig. 2 Linkage disequilibrium (*LD*) analysis of *DPYD*. Pairwise LD between 18 common SNPs (>0.01 in allele frequencies) is expressed as r^2 (*upper*) and |D'| (*lower*) by a 10-graded *blue color*. The *denser color* indicates higher linkage. The haplotype block partition based on LD measure |D'| of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

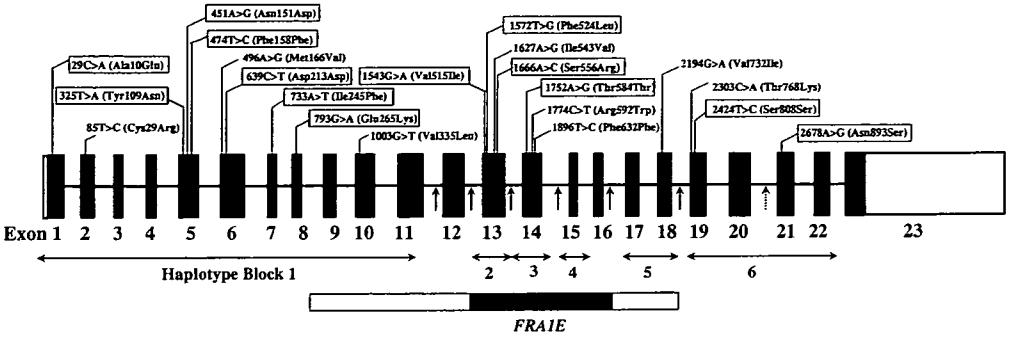

Fig. 1 Twenty-one variations detected in the coding exons are depicted in the schematic diagram of the *DPYD* gene. Fourteen novel variations are enclosed by *squares*. The recombination spots were estimated based on the LD profiles obtained from Japanese data in the HapMap project and indicated by *arrows*. 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[e]		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**C/T**TCCCCTCCCCGC			0.021
MPJ6_DPD002[a]		5′-Flank	52206348	–477	TTGAGGAGTTCC**T/G**GAAAATGCAGTT			0.026
MPJ6_DPD003[a]		5′-Flank	52206137	–266	CTCCCTCCCTCCC**C/A**TTCTGCTTGCAG			0.045
MPJ6_DPD004[a]		5′-Flank	52206114	–243	AGGCTGGGGCGC**G/A**GAGCGGGCTGAA			0.0059
MPJ6_DPD005[a]		Exon 1	52205843	29	GTAAGGACTCGG**C/A**GGACATCGAGGT	Ala10Glu		0.0015
MPJ6_DPD006[g]	rs1801265	Exon 2	52168278	85	CATGCAACTCTG**T/C**GTTCCACTTCGG	Cys29Arg	*9	0.029
MPJ6_DPD007[a]		Intron 2	52168055	IVS2 + 158	TTTGAAAGTGTA**T/C**TTTTAATTACAC			0.0015
MPJ6_DPD008[a]		Intron 3	52113040	IVS3 + 23	GTCACCATAGCA**A/G**CAGTCACAGATG			0.0029
MPJ6_DPD009[a]		Exon 5	52006617	325	ATTTTGCAGAAC**T/A**ATTATGGAGCTG	Tyr109Asn		0.0029
MPJ6_DPD010[a]		Exon 5	52006491	451	GAGGGACCCATT**A/G**ATATTGGTGGAT	Asn151Asp		0.0088
MPJ6_DPD011[a]		Exon 5	52006468	474	ATTGCAGCAATT**T/C**GCTACTGAGGTA	Phe158Phe		0.0044
MPJ6_DPD012[a]		Intron 5	51984611	IVS5–115	CATATTAATACT**G/A**AAAATGTACTGC			0.021
MPJ6_DPD013[g]	rs2297595	Exon 6	51984484	496	GTATTCAAAGCA**A/G**TGAGTATCCCAC	Met166Val		0.022
MPJ6_DPD014[a]		Exon 6	51984341	639	GGGGTACTCTGA**C/T**ATCACTATATTT	Asp213Asp		0.0088
MPJ6_DPD015[a]		Exon 7	51976695	733	GTGAATTTTGAG**A/T**TTGAGCTAATGA	Ile245Phe		0.0015
MPJ6_DPD016[a]		Intron 7	51976602	IVS7 + 64	CTCTACACTAAA**G/T**ATTAACAGCAAA			0.0015
MPJ6_DPD017[a]		Exon 8	51964101	793	CTTTCAGTGAAT**G/A**AAATGACTCTTA	Glu265Lys		0.0015
MPJ6_DPD018[a]		Intron 8	51963953	IVS8 + 91	TTCAGACATTTT**C/T**GTGATGAAAGTT			0.0088
MPJ6_DPD019[a]		Intron 9	51878456	IVS9–120	TTTGATAGTGAC**A/T**CTTCATCCTGGA			0.0029
MPJ6_DPD020[b]		Exon 10	51878292	1003	ATACGGGGAGTC**G/T**TGATTGTACTTG	Val335Leu	*11	0.0015
MPJ6_DPD021[a]		Intron 10	51878143	IVS10 + 24	CCATCAGAAAAT**A/G**TGGAGTTGTACT			0.0015
MPJ6_DPD022[c]		Intron 10	51858934	IVS10-15	TTTCTTCTCTGT**T/C**CTGTTTTGTTTT			0.018
MPJ6_DPD023[a]		Intron 12	51800901	IVS12-11	AAGTATTGGTTT**G/A**TATTTTGCAGTC			0.038
MPJ6_DPD024[a]		Intron 12	51800899	IVS12-9	GTATTGGTTTGT**A/G**TTTTGCAGTCAC			0.0073
MPJ6_DPD025[a]		Exon 13	51800872	1543	TATGGAGCTTCC**G/A**TTTCTGCCAAGC	Val515Ile		0.0015
MPJ6_DPD026[a]		Exon 13	51800843	1572	ACTACCCCTCTT**T/G**TACACTCCTATT	Phe524Leu		0.0015
MPJ6_DPD027[g]	rs1801159	Exon 13	51800788	1627	GGATTGAAGTTT**A/G**TAAATCCTTTTG	Ile543Val	*5	0.283
MPJ6_DPD028[a]		Exon 13	51800749	1666	ACTCCAGCCACC**A/C**GCACATCAATGA	Ser556Arg		0.0015
MPJ6_DPD029	rs2786783	Intron 13	51800636	IVS13 + 39	AGAAATGTCTATC**T/C**TATATATTTTAAT			0.283
MPJ6_DPD030	rs2811178	Intron 13	51800635	IVS13 + 40	GAAATGTCTATC**G/A**TATATTTTAATT			0.179
MPJ6_DPD031[a]		Intron 13	51735220_51735219	IVS13-47_-48	ATAAAGATTATA**-/TA**GCTTTTCTTTGT			0.0015
MPJ6_DPD032[a]		Exon 14	51735161	1752	GGACATTGTGAC**A/G**AATGTTTCCCCC	Thr584Thr		0.0015
MPJ6_DPD033[f]		Exon 14	51735139	1774	CCCAGAATCATC**C/T**GGGGAACCACCT	Arg592Trp		0.0015
MPJ6_DPD034[g]	rs17376848	Exon 14	51735017	1896	AAAGGCTGACTT**T/C**CCAGACAACGTA	Phe632Phe		0.139
MPJ6_DPD035[a]		Intron 14	51734989	IVS14 + 19	GTGATTTAACAT**C/A**TAAAACAAGAGA			0.0088
MPJ6_DPD036[a]		Intron 14	51734908	IVS14 + 100	TTAATGTGTATA**T/G**TTTATTAAAGAA			0.0015
MPJ6_DPD037[a]		Intron 14	51667533	IVS14-123	GATTTATTTTTT**C/A**ACAGTTTGAAAA			0.155
MPJ6_DPD038[a]		Intron 14	51667431	IVS14-21	TGAACTTATATT**C/A**TTTTGTTTTTCT			0.0015
MPJ6_DPD039[d]		Intron 15	51667267	IVS15 + 75	TAAAGAGCTGCC**A/G**TGAGAAATAATA			0.155
MPJ6_DPD040[a]		Intron 16	51591373	IVS16-127	GGAATTTGAGAA**A/G**TATATCATGTAG			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 (Phe524Leu), 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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