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Germline Mutation of Dihydropyrimidine Dehydrogenese Gene among a Japanese Population in Relation to Toxicity to 5-Fluorouracil

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5-Fluorouracil (5FU) is most commonly used in chemotherapy for human malignancy. Over 80% of administered 5FU is metabolically degraded by dihydropyrimidine dehydrogenase (DPD), a primary and rate-limiting enzyme in the 5FU metabolic pathway. A DPD-deficient phenotype among cancer patients, which has posed a serious problem in 5FU-based chemotherapy, was reported to be in part ascribed to germline mutations in dihydropyrimidine dehydrogenase (*DPYD*) gene. Therefore, we for the first time examined the frequencies and types of germline mutations in the *DPYD* gene among a total of 107 Japanese cancer patients and healthy volunteers. Of 214 alleles examined among them, 181 alleles were of the same type, which was assigned as wild type; 21 alleles revealed a nucleotide substitution resulting in silent mutation; and the remaining 12 alleles showed five types of nucleotide deletion or substitutions resulting in one frameshift and four missense mutations. Three of them, A74G, 812delT and L572V, were novel mutations. None of the study subjects showed homozygous frameshift or missense mutated alleles. We also studied the association between toxic response to 5FU and heterozygous frame shift or missense mutation of the *DPYD* gene among eight cancer patients who had received 5FU-based chemotherapy. These patients did not show any adverse effects higher than grade 3, suggesting that heterozygotes are not associated with increased toxicity to 5FU. Our results indicate that a very small percentage, about 0.2%, of the Japanese population seems to carry homozygous mutations in *DPYD* gene, mutations which possibly indicate genetically increased toxicity of 5FU-based chemotherapy.

Key words: Dihydropyrimidine dehydrogenase gene — Germline mutation — 5FU-based chemotherapy

Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is a primary and rate-limiting enzyme in pyrimidine base catabolism, and it is also known to be responsible for the metabolic degradation of 5-fluorouracil (5FU), a major chemotherapeutic agent used for the treatment of various cancers. Since more than 80% of administered 5FU is degraded *in vivo* by DPD to fluorinated β-alanine, the level of DPD catalytic activity affects the efficacy or toxicity of 5FU. The DPD activity is found in various human tissues with the highest activity observed in hepatocytes and peripheral blood mononuclear cells.[1)]

DPD deficiency is reported to be associated with various clinical phenotypes of toxicity: specifically, cancer patients with DPD deficiency sometimes exhibit severe toxicity to 5FU-based chemotherapy.[2, 3)] A small fraction of DPD deficiency may be generated by the use of 5FU combined with drugs such as sorivudine, which inhibits DPD activity.[4)] However, recent studies on the molecular genetics of DPD deficiency indicated a close association between DPD-deficient phenotypes and types of genetic aberrations in dihydropyrimidine dehydrogenase (*DPYD*) gene, such as exon skipping,[5)] deletion,[6)] frameshift and missense mutation.[7–9)] Furthermore, homozygous carriers with *DPYD* mutated alleles exhibited much severer toxic responses to 5FU treatment than those with wild alleles.[10)] Thus, the critical questions in 5FU-based chemotherapy appeared to be these: 1) How common are germline mutations in the *DPYD* gene among cancer patients? 2) Would heterozygous carriers, who are expected to be more frequent than homozygous ones, show increased toxicity in response to 5FU treatment, compared with carriers of wild alleles?

Therefore, in this paper we report for the first time the frequency and types of germline mutations in the *DPYD* gene among a Japanese population composed of both cancer patients and healthy volunteers, and we describe the toxic response of the heterozygous carriers to 5FU treatment.

MATERIALS AND METHODS

cDNA synthesis of *DPYD* gene Blood samples were collected from a total of 107 Japanese individuals: 104 cancer patients admitted to Saitama Cancer Center Hospital and three healthy volunteers. Of these, 69 of the patients had received 5FU-based chemotherapy. Total RNA of each sample was isolated from peripheral blood mononuclear

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cells; cDNA was synthesized using a "GeneAmp" RNA PCR Core Kit (Perkin-Elmer, Foster City, CA). This study was permitted by the Ethics Committee of Saitama Cancer Center, under the condition that all personal information was deleted from study data, and with the informed consent of all study subjects.

PCR SSCP and sequencing We performed PCR SSCP to identify aberrations in the *DPYD* gene. PCR was carried out at a final concentration of 1× PCR buffer (10× PCR buffer; 150 m*M* Tris-HCl, pH 8.0, 500 m*M* KCl), 2–3 μl of RT product, 2.5 m*M* $MgCl_2$, 0.5 μM each 5′ or 3′ primer, and 1.25 U of "AmpliTaq Gold" (PE Applied Biosystems, Branchburg, NJ) in a total volume of 50 μl. All PCR started at 95°C for 9 min, followed by 40 cycles of 30 s at 65°C and 30 s at 94°C. The primers used in PCR are summarized in Table I. Primer sets in Table Ia were used to analyze splicing variants. PCR products (5 μl each) were subjected to electrophoresis (1% agarose gel) and visualized with ethidium bromide staining. Subsequently, PCR products obtained from primer sets DPD1L, 2L, 3L and 4L were digested with either *Alu*I or [*Rsa*I plus *Sac*I], *Alu*I or [*Hind*III plus *Sac*I], *Alu*I or *Hinf*I, and *Alu*I or *Rsa*I, respectively, resulting in adequate lengths of DNA fragments for subsequent non-radioisotopic SSCP.[11] To avoid possible failure in detecting mutations, we used other sets of primers (Table Ib), which divided *DPYD* cDNA into 13 fragments. PCR products that showed abnormal mobility shifts in non-radioisotopic SSCP were subjected to bidirect sequencing using an Applied Biosystems model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT). To confirm the sequence aberrations, we carried out genomic DNA analysis using peripheral blood mononuclear cells as described previously.[12]

Table I. Primer Sets Used for RT-PCR SSCP

a)	
DPD1L-F	5′-GAGGGTTTGTCACTGGCAGA-3′
DPD1L-R	5′-GCTCCAAGTACAATCACGAC-3′
DPD2L-F	5′-TGCCAGAACCCAATAAAGAT-3′
DPD2L-R	5′-AGTCAGCCTTTAGTTCAGTG-3′
DPD3L-F	5′-GTGACAAATGTTTCCCCCAG-3′
DPD3L-R	5′-TTCCAGATAAGGTCCAAAAC-3′
DPD4L-F	5′-GAACTACAAGACTGGGATGG-3′
DPD4L-R	5′-TCCTTGGATACATTTTCTTG-3′
b)	
DPD1F	5′-TTGAGGACGCAAGGAGGGTTTG-3′
DPD1R	5′-GACATACCATTCCACAAGTCAGACC-3′
DPD2F	5′-AAATGTGCAGATGCCCCGTG-3′
DPD2R	5′-AGTGATGTCAGAGTACCCCAATCG-3′
DPD3F	5′-TTGCTCTTTTTGGTGCTGGG-3′
DPD3R	5′-CGCACATTCCTGCTTTACTGC-3′
DPD4F	5′-CGCAGGACCAGGGGTTTTATAC-3′
DPD4R	5′-ACTGATGACCACATCGGCTTTC-3′
DPD5F	5′-TGTTGCTATGCAGTTTGTTCGG-3′
DPD5R	5′-TAAAAGAGGGGTAGTTCAGGCTTG-3′
DPD6F	5′-TACAGTCACAATATGGAGCTTCCG-3′
DPD6R	5′-TTTGACACCAATATGCAGCCG-3′
DPD7F	5′-GTTTCCCCCAGAATCATCCG-3′
DPD7R	5′-GCATCTGCTCCAGAATCCTCAG-3′
DPD8F	5′-GGCTGACTTTCCAGACAACATTG-3′
DPD8R	5′-CATTGGCACCACCTTCCTTTG-3′
DPD9F	5′-GGGTTAGGCAAGCTGTTCAGATTC-3′
DPD9R	5′-GGAAGCACCACTATGGAGAAACTG-3′
DPD10F	5′-GGCTACTGGTGGAATTGACTCTGC-3′
DPD10R	5′-CCAGATAAGGTCCAAAACTTGGC-3′
DPD11F	5′-ACCAGAAAGGGAAACCAGTTCC-3′
DPD11R	5′-ACAAGTGTCGGTTATGGTGGGC-3′
DPD12F	5′-CAACGTAGAGCAAGTTGTGGCTATG-3′
DPD12R	5′-GGTGACATGAAAGTTCACAGCAAC-3′
DPD13F	5′-TTCCAGGACAACACCTTATGAACC-3′
DPD13R	5′-AAATATGGAGCACAGCATAGGGC-3′

5FU-based chemotherapy In methotrexate (MTX)-5FU, 5FU (800 mg/m^2) was given intravenously for 1 h by drip infusion after MTX (100 mg/m^2) intravenous injection; at 24 h after 5FU administration, calcium folinate (21 mg × 6 times each 6 h) was given orally. In cisplatin (CDDP)-5FU, 5FU (500 mg/m^2/day) was administered by continuous intravenous infusion on days 1 to 5; CDDP (10 mg/m^2) was administered intravenously by 2 h drip infusion on days 1 to 5; before the administration of CDDP, 5-HT3-receptor antagonist was orally or intravenously administered. This regimen was repeated bi- or tri-weekly. In 5FU-leucovorin (LV), 5FU (500 mg/m^2) was continuously administered by intravenous infusion on days 1 to 5; calcium folinate (20 mg/m^2) was also given in 1 h drip infusion on days 1 to 5. The World Health Organization (WHO) standard criteria[13] were used for the evaluation of toxic or effective response to 5FU.

RESULTS

Germline mutations of the *DPYD* gene We examined germline mutations of the *DPYD* gene in 214 alleles from 107 cancer patients and healthy volunteers. Of these, 181 alleles were the same type and were assigned as the wild type; 33 alleles revealed genetic aberrations from the wild type, 21 alleles with a nucleotide substitution at codon 632 that resulted in silent mutation, and 12 alleles with a nucleotide substitution or deletion that resulted in a different protein structure (Table II). The localization of these mutations in *DPYD* cDNA is shown in Fig. 1.

The silent mutation of T1896C in codon 632 was found in homozygous alleles of one subject (TTC/TTC) and in heterozygous alleles (TTC/TTT) of 19 subjects: an allelic frequency of 9.8% (21/214) for TTC, which is in accordance with the Hardy Weinberg equilibrium. All missense and frameshift mutations were found in heterozygous

alleles of 12 subjects, and the five types found included one frameshift and four missense mutations (Table II): the allelic frequency of these mutations was 5.6% (12/214). The mutations of A74G in codon 25 (H25R), 812delT in codon 271 and C1714G in codon 572 (L572V) were found in one subject, mutations reported for the first time in this study; the mutation of T85C in codon 29 (C29R) found in 8 patients has already been reported in DPD-deficient patients[7, 8, 14, 15]; the mutation of A1627G in codon 543 (I543V) found in one patient was reported to generate normal DPD activity.[15–17] A typical pattern of SSCP and result of direct sequencing are shown in Figs. 2 and 3.

These mutations were confirmed using genomic DNA extracted from peripheral blood mononuclear cells, except in two C29R mutations and one H25R mutation, where the peripheral blood was not available. The mutations of cDNA and genomic DNA examined were identical.

Toxic response in patients with *DPYD* gene mutations
None of the 107 study subjects had any past history of convulsion, motor or mental retardation, or other symptoms which might be associated with DPD deficiency. Of 12 patients with heterozygously missense or frameshift mutated alleles, 8 had received 5FU-based chemotherapy. None of the eight patients had shown a toxic response over grade 2 (Table III) the most common adverse effect being nausea of grade 1 or 2. On the other hand, of 61 patients with wild or silently mutated alleles who had received 5FU-based chemotherapy (MTX-5FU for 43 patients, CDDP-5FU for 16 patients and CDDP-5FU plus radiation for two patients), adverse effects of grade 3 and over had been observed in 11 patients (leucocytopenia in eight patients, leucocytopenia plus diarrhea in one patient, nausea in one patient, thrombocytopenia in one patient, and arrhythmia in one patient). There was no substantial

Table II. Mutations of *DYPD* Gene Found in 107 Japanese Cancer Patients and Healthy Volunteers

Exon	Codon	Nucleotide (amino acid) change		Frequency	Mutation	Activity
2	25	A74G (H25R)	CAT (His)–CGT (Arg)	1/214 (0.46%)	missense	n.r.
2	29	T85C (C29R)	TGT (Cys)–CGT (Arg)	8/214 (3.7%)	missense	low *in vivo*[a]
8	271	812delT	ACTTTG–ACTTGA	1/214 (0.46%)	frameshift	n.r.
13	543	A1627G (I543V)	ATA (Ile)–GTA (Val)	1/214 (0.46%)	missense	normal[b]
13	572	C1714G (L572V)	CTC (Leu)–GTC (Val)	1/214 (0.46%)	missense	n.r.
14	632	T1896C (F632F)	TTT (Phe)–TTC (Phe)	21/214 (9.8%)	silent	n.r.

a) Vreken *et al.*[7, 14]
b) Ridge *et al.*[16]
n.r., not reported.

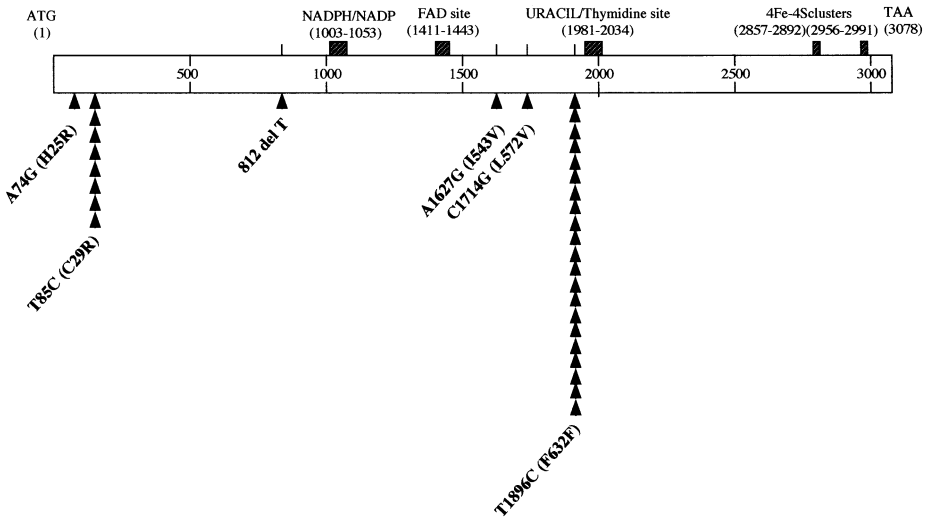

Fig. 1. Germline mutations in *DPYD* cDNA. Arrowheads indicate the mutations found in 107 study subjects, at the positions described above the figure; closed boxes represent the conserved motifs including putative binding sites.

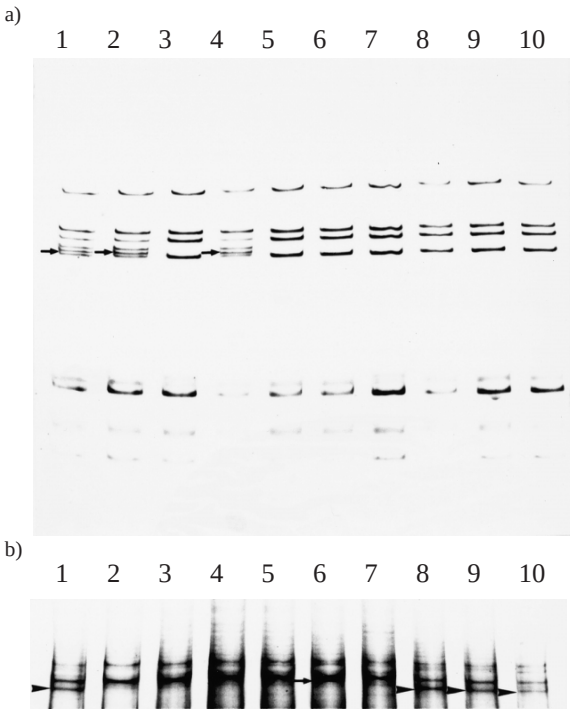

Fig. 2. a) Non-radioisotopic SSCP of the DPD3L product digested by *Alu*I. Mobility shift from C1896 is indicated with arrows. b) Non-radioisotopic SSCP of the DPD1 product. Mobility shifts from T85C (C29R) are indicated with wedges, and from A74G (H25R), with arrows.

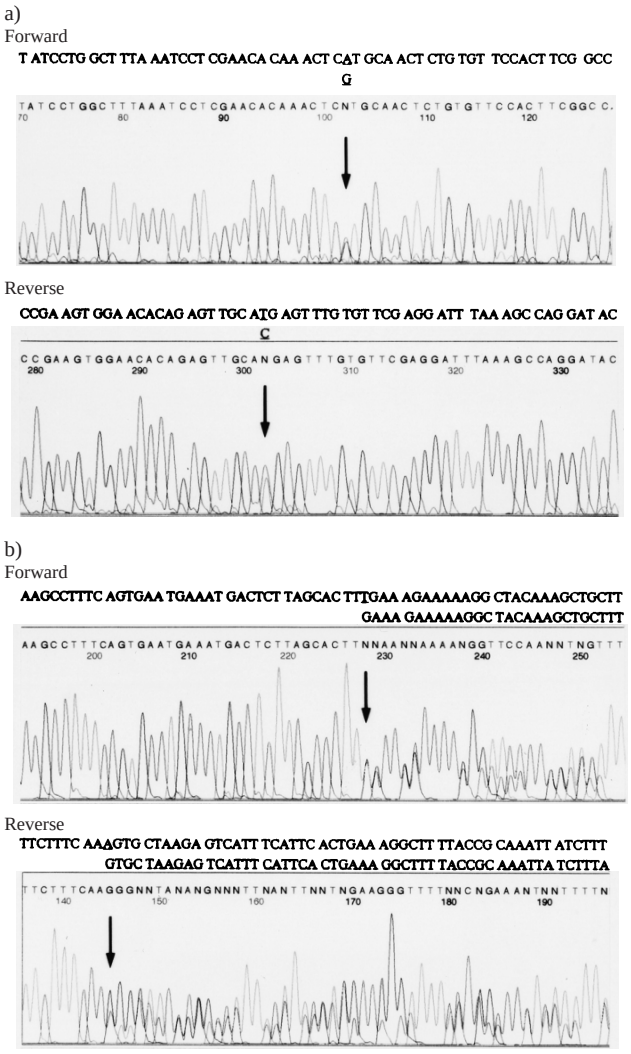

Fig. 3. Identification of a) the A74G (H25R) and b) 812delT mutation. Direct sequence analysis was performed using amplified *DPYD* cDNA fragments. Nucleotide substitution A74G and deletion 812delT are indicated with an arrow.

difference in dose or duration of 5FU-based chemotherapy for patients with or without mutations. Since the frequency of adverse effects of grade 3 and over in the latter group was 18%, patients with heterozygously mutated alleles of *DPYD* gene showed no excess toxicity to 5FU treatment when compared with those having wild or silently mutated alleles.

Table III. *DPYD* Gene Mutations and Toxicity of 5FU-based Chemotherapy

Mutation	Cancer	Sex	Chemotherapy	Toxicity	Grade	Efficacy
A74G (H25R)	gastric	female	MTX-5FU	nausea	1	p.d.
T85C (C29R)	gastric	female	MTX-5FU	nausea	1	p.d.
T85C (C29R)	gastric	female	MTX-5FU	nausea	2	n.e.
T85C (C29R)	gastric	male	MTX-5FU	nausea	2	n.e.
T85C (C29R)	rectal	male	5FU-LV	no	0	n.c.
812delT	colon	male	MTX-5FU	nausea	1	p.d.
A1627G (I543V)	esophageal	male	CDDP-5FU+Radiation	nausea	2	p.r.
C1714G (L572V)	gastric	female	MTX-5FU	arrhythmia	2	p.d.

p.d., progressive disease; n.e., not evaluable; n.c., no change; p.r., partial response.

DISCUSSION

Analysis of genetic polymorphism of drug-metabolizing enzymes is thought to be a useful tool in predicting the efficacy of chemotherapy and the likelihood of adverse effects on individual patients. We thus focused on genetic polymorphisms in the *DPYD* gene, which may influence the metabolic pathway of 5FU in cancer patients. We found that the frequency of mutated alleles which resulted in differing protein structures through missense and frameshift mutations was 5.6% (12 heterozygous carriers in a total of 107 study subjects), with three novel types of mutation revealed. Heterozygous carriers did not show any obvious excess in toxic response to 5FU-based chemotherapy when compared with those without mutated alleles.

Although homozygous carriers were not found among our study subjects, we can estimate the frequency of homozygous carriers assuming the Hardy Weinberg equilibrium (which was the case for silent mutation of T1896C): the frequency of homozygous A74G, 812delT, A1627G or C1714G will be 2.1 in 10^5 persons and that of T85C will be 1.4 in 10^3 persons. Of these mutations, T85C has been reported to cause decreased DPD activity,[7, 8, 14, 15] 812delT is thought to reduce DPD activity, since this deletion is located upstream of the 1897delC known to cause loss of activity.[16] Combining these frequencies, a very small percentage, about 0.2%, of the Japanese population is predicted to carry homozygously mutated alleles in the *DPYD* gene that may result in genetically increased toxicity of 5FU-based chemotherapy. It was also of interest that our study subjects did not show the 165 base pair exon skipping mutation most commonly found in association with low DPD activity in a European population.[16] This suggests a large racial difference in frequency and type of mutations in the *DPYD* gene, along with the possibility of a genotype-phenotype association, supporting the significance of our study in a Japanese population.

The remaining critical question, mentioned earlier, is whether heterozygous carriers exhibit increased toxicity to 5FU-based chemotherapy. In fact, reduction of DPD activity by 50% in cancer patients caused a severe toxic response to 5FU,[10] and the low DPD activity of some patients has been in part ascribed to a heterozygously mutated allele in the *DPYD* gene.[17, 18] In addition, only 17% of reduced DPD activity had a molecular basis for the deficient phenotype.[19] However, our observations of Japanese patients implied that the heterozygote is not associated with increased toxic response to 5FU, although a further investigation with a larger number of patients will be necessary to confirm our findings.

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