Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme catalyzing the metabolic degradation of the anticancer drug 5-fluorouracil (5-FU). Population studies of DPD activity in peripheral blood mononuclear cells (PBMC) were reported in healthy volunteers and cancer patients. Although these studies were done in mainly Caucasian and African American populations, only a little information is available for a Japanese population.

**Abstract**

**Purpose:** Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme catalyzing the metabolic degradation of the anticancer drug 5-fluorouracil (5-FU). Population studies of DPD activity in peripheral blood mononuclear cells (PBMC) were reported in healthy volunteers and cancer patients. Although these studies were done in mainly Caucasian and African American populations, only a little information is available for a Japanese population.

**Experimental Design:** One hundred fifty healthy Japanese volunteers were screened for a population distribution of PBMC-DPD activity. Genetic analysis of a volunteer with very low DPD activity was carried out by reverse transcriptase-PCR and genomic sequencing. Bacterially expressed recombinant mutant DPD proteins were purified and characterized.

**Results:** Mean and median values of PBMC-DPD activity for 5-FU reduction in the study population were 0.173 and 0.166 nmol/min/mg protein, respectively. A 57-year-old female volunteer (proband in this study) had very low DPD activity (0.014 nmol/min/mg protein) with a very low level of expression of DPD protein. Two novel nucleotide substitutions, at nucleotide positions 1097 (1097G>C) and 2303 (2303C>A), resulting in amino acid substitutions at positions 366 (G366A) and 768 (T768K), respectively, were identified. The G366A mutation caused not only a marked decrease in the affinity of the enzyme to cofactor NADPH but also reduced $V_{max}$ for 5-FU-reducing activity to ~0.5. T768K mutant lost its activity much faster than did wild DPD.

**Conclusions:** We found one healthy volunteer (0.7% of the population) with very low PBMC-DPD activity due to heterozygosity for a mutant allele of the $DPYD$ gene in a population of 150 Japanese.

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isolated, only little information is available for a Japanese population. For example, Kouwaki et al. (23) reported mean PBMC-DPD activity in 10 healthy adult Japanese volunteers. We also showed liver cytosolic DPD activities of 21 Japanese cancer patients (12).

The present study deals with (a) a population distribution of PBMC-DPD activity in 150 Japanese healthy volunteers, (b) genetic analysis of a female volunteer with very low DPD activity, and (c) characterization of bacterially expressed mutant DPD proteins.

Materials and Methods

Materials. [14C]5-FU (2.1 MBq/μmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Restriction enzymes, T4 DNA ligase, and EX Taq polymerase were purchased from TaKaRa Bio, Inc. (Shiga, Japan); ampicillin (Na), NADPH, isopropl-β-D-thiogalactopyranoside, Triton X-100, and uracil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Xpress protein expression system (pTrcHis) and ProBond resin were from Invitrogen Co. (San Diego, CA). 2', 5'-ADP-Sepharose 4B and a PD-10 column were obtained from Amersham Pharmacia Biotech UK, Ltd. (Amersham Place, England) and molecular weight markers from Sigma Chemical Co. (St. Louis, MO). Other reagents used were of analytic grade.

Healthy volunteers. One hundred fifty healthy Japanese volunteers (75 males and 75 females; mean age, 46 years) participated in this study (see details in Table 1). The paid volunteers were selected so that age distribution did not differ significantly between male and female subjects (P = 0.70). The proband, found among the 150 volunteers by measuring their PBMC-DPD activity, was a 57-year-old female who was apparently healthy. Her DPD activity was 8% of the mean value for the entire study population. Available members of the proband’s family, a son and daughter, also participated in addition to the 150 volunteers. Their data were not included in the statistical analysis. Written informed consent was provided by each volunteer. The present study was approved by the ethics committee of the School of Medicine, Gifu University.

Isolation of RNA and reverse transcriptase-PCR. Total RNA extraction from PBMC and amplification of DPD cDNA by reverse transcriptase-PCR (RT-PCR) were done using ISOGEN (Nippon Gene, Tokyo, Japan) and TaKaRa RNA PCR kits (TaKaRa Bio), respectively, according to the manufacturers’ instructions. The entire coding region of DPD cDNA was amplified in three overlapping fragments, F1 (1.2 kb), F2 (1.2 kb), and F3 (1.3 kb), with primer pairs designed based on the reported cDNA sequence of human DPD (25). Sequences of PCR primers for the amplification of DPD cDNA are shown in Table 2. PCR condition was as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes. The amplified products were isolated by agarose gel electrophoresis and analyzed by direct sequencing using sequence primers designed from internal sequences of the fragments. Sequence determination was carried out using an ABI377 DNA sequencer (Applied Biosystems, Foster City, CA) with a dRhodamine dye-terminator cycle sequencing kit (Applied Biosystems).

Genotyping of the mutant DPDP allele. Genomic DNA was isolated from 10 μl of whole blood by the Single-Tube PCR Kit (TaKaRa Bio) according to the manufacturer’s instructions. Isolated genomic DNA was used as a template for amplification of exons 10 and 19 with exon-specific primers reported by van Kuilenburg et al. (26). PCR condition was as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR product was isolated by agarose gel electrophoresis and sequenced directly using sequencing primers designed from exon sequences.

Bacterial expression and purification of wild and mutant dihydropyrimidine dehydrogenase proteins. The expression plasmid for the wild type of DPD was constructed by a previously described method (11). The resultant construct, designated pTrcHis(hDPD), encoded the full-length human DPD subunit with an NH2-terminal fusion peptide of imidine dehydrogenase proteins.

PCR-based mutagenesis was used for the introduction of mutations (1097G > C and/or 2303C > A) into the DPD coding sequence by using restriction sites XhoI, CmI, NdeI, and EcoRI. The result mutant clones were sequenced to confirm the introduction of mutations and were designated pTrcHis(hDPD1097G > C), pTrcHis(hDPD2303C > A), and pTrcHis(hDPD1097G > C/2303C > A), respectively. These expression plasmids were independently transfected into Escherichia coli TOP10. Cultures (1 liter) of the transformed bacteria were grown at 30°C in Luria-Bertani medium containing 1 mmol/L uracil, 100 μg/mL ampicillin, 100 μmol/L each of FAD and FMN, and 10 μmol/L each of Na2S and Fe(NH4)2SO4 to an absorbance at 600 nm of 1.2 in the absence of isopropl-β-D-thiogalactopyranoside and then at 25°C for 9 hours, following the addition of isopropl-β-D-thiogalactopyranoside (1 mmol/L). Cytosol from the bacterial cells harvested from the culture was prepared as previously shown (11). Wild and mutant types of DPD proteins were purified by chromatography on ProBond Ni-binding resin and 2',5'-ADP Sepharose 4B affinity columns as described previously (11).

SDS-PAGE and immunoblot analyses. SDS-PAGE was carried out on 7.5% to 15% polyacrylamide gradient gel plates by the method of

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (y), mean ± SD (range)</th>
<th>Weight (kg), mean ± SD (range)</th>
<th>Height (cm), mean ± SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>150</td>
<td>46.1 ± 16.7 (21-81)</td>
<td>58.8 ± 11.3 (34.4-109.0)</td>
<td>161.3 ± 9.3 (139.2-187.8)</td>
</tr>
<tr>
<td>Men</td>
<td>75</td>
<td>45.5 ± 16.5 (21-81)</td>
<td>64.9 ± 9.0 (44.0-87.7)</td>
<td>167.7 ± 7.2 (151.7-187.8)</td>
</tr>
<tr>
<td>Women</td>
<td>75</td>
<td>46.7 ± 16.9 (23-81)</td>
<td>52.7 ± 10.0 (34.4-109.0)</td>
<td>155.0 ± 6.2 (139.2-170.3)</td>
</tr>
</tbody>
</table>

NOTE: Age distribution was not significantly different between genders (P = 0.70).

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Laemmli (27) and immunoblot by the method of Towbin et al. (28). DPD protein was detected in cytosolic fractions from human liver and PBMC using the Enhanced Chemiluminescence Western Blotting Analysis System (Amersham Pharmacia Biotech UK) with rabbit antiserum raised against human DPD (11) as the primary antibody. Human liver cytosol was prepared from a liver obtained by partial hepatectomy as reported previously (13). The liver cytosolic 5-FU reducing activity was 420 pmol/min/mg protein.

Enzyme assay. DPD activity of PBMC cytosol and purified DPD was assayed as reported previously using [14C]-5-FU (9) as a substrate. In brief, the enzyme source (5 µL) was incubated with 20 µmol/L [14C]-5-FU (2.1 MBq/µmol) at 37°C for 5 minutes in the presence of 200 µmol/L NADPH, 2.5 mmol/L MgCl2, and 10 mmol/L 2-mercaptoethanol in a final volume (50 µL) of 30% (v/v) glycerol/35 mmol/L K-phosphate buffer (pH 7.4). The reaction mixture was preincubated at 37°C for 1 minute, and the reaction was initiated by the addition of the substrate. For thermal stability experiments, purified DPD proteins of the wild type or three types of mutants were preincubated at 45°C for various times in the absence of NADPH and 5-FU before assay. Kinetic studies of the reduction of 5-FU were done at various substrate concentrations, ranging from 0.5 to 200 µmol/L, in the presence of 200 µmol/L NADPH. To study the dependence on NADPH concentration, the cofactor was used at concentrations of 0.5 to 1000 µmol/L in the presence of 20 µmol/L 5-FU. Apparent Kₘ and Vₘax values were estimated by graphic extrapolation of the data in double reciprocal plots obtained in the zero-order kinetics region of the enzymatic reactions. Data were obtained from at least three experiments.

Statistical analysis. All statistical calculations were carried out using SAS 8.2 software (SAS Institute, Inc., Cary, NC). The mean, SD, and range of PBMC-DPD activity were determined. The differences among the groups by sex and age were analyzed by the Student’s t test or one-way factorial ANOVA test. In case of significant result after ANOVA, intergroup comparisons were done by post hoc test. Statistical significance was assigned to Ps < 0.05. Correlations between age and DPD activity were assessed by simple linear regression analysis.

Results

Population distribution of peripheral blood mononuclear cells-dihydropyrimidine dehydrogenase activity. PBMC-DPD activities in 150 healthy Japanese volunteers showed a unimodal distribution and globally followed a Gaussian distribution (Fig. 1). Mean and median activity values for the entire population were 0.173 and 0.166 nmol/min/mg protein, respectively (Table 3). A large degree of interindividual variation was observed; maximum and minimum activities respectively (Table 3). A large degree of interindividual variation was observed; maximum and minimum activities were 0.371 and 0.014 nmol/min/mg protein, respectively, with an SD value of 0.055. Mean values of DPD activity in males and females were 0.181 ± 0.058 and 0.165 ± 0.052 nmol/min/mg protein, respectively. However, no statistically significant difference was observed according to gender. Analysis according to age group showed that DPD activity in those older than 65 years of age was significantly higher than in the younger age groups (P < 0.05). Correlation analysis between age and DPD activity in the total study population showed a weak but statistically significant positive correlation (r = 0.346, P < 0.01; Fig. 2). There was no relationship between DPD activity and height or weight of the subjects.

Phenotyping of the proband. A 57-year-old woman volunteer (proband in this study) with no apparent clinical or physiologic abnormality had very low PBMC-DPD activity (0.014 nmol/min/mg protein), which was only 8% of the mean activity of the study population. To exclude the possibility of technical error, a second blood sample was collected and PBMC-DPD activity was redetermined. The second determination still revealed very low activity (0.008 nmol/min/mg protein). PBMC cytosol from her children, a son and daughter, showed 5-FU reducing activity of 0.092 and 0.095 nmol/min/mg protein, respectively, which were calculated as 53% and 55%, respectively, of the mean activity of the study population. Urinary uracil and creatinine concentrations of the proband were

![Graph showing population distribution of PBMC-DPD activity in 150 healthy Japanese volunteers. Statistical analysis showed that the activity satisfactorily fit the Gaussian distribution.](image-url)

Table 2. Primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence</th>
<th>Nucleotide positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1-F</td>
<td>5'-GTTTTGTCTACTTGCGAGACTCAGAATGAC-3'</td>
<td>–38 to –9</td>
</tr>
<tr>
<td>F1-R</td>
<td>5'-CTTCTCTAGCGAAGCTCATCCTCCTCCAGG-3'</td>
<td>1120-1148</td>
</tr>
<tr>
<td>F2-F</td>
<td>5'-ACCTGTAGCCAAAGCGTAAAGGCTTAGGATG-3'</td>
<td>933-963</td>
</tr>
<tr>
<td>F2-R</td>
<td>5'-CTGAAACGCTTGCCCTACCCAGGCGAGA-3'</td>
<td>2081-2109</td>
</tr>
<tr>
<td>F3-F</td>
<td>5'-CAACATGTGATGTGCAATGATGATGAGC-3'</td>
<td>1902-1931</td>
</tr>
<tr>
<td>F3-R</td>
<td>5'-GCCACTAATTTGAATGTGAGTGCATGACAG-3'</td>
<td>3241-3269</td>
</tr>
</tbody>
</table>

*Primers used for amplification of DPD cDNA in three overlapping fragments, F1, F2, and F3, as indicated in Materials and Methods. “-F” and “-R” in the primer names indicate sense and antisense primers, respectively.

*Corresponding nucleotide positions on DPD cDNA reported by Yokota et al (25).
DPYD sequence. were shown to be heterozygous with respect to the wild-type T768K), respectively. As indicated in Fig. 4A, these mutations nated G366A) and 768 (from threonine to lysine, designated substitutions at positions 366 (from glycine to alanine, desig-
shown the existence of two novel nucleotide substitutions, at positions 1097 (from G to C, designated 1097G > C) and 2303
amplified in three overlapping fragments with lengths of 1,157, 1,177, and 1,368 bp. Sequence analysis of these fragments showed the existence of two novel nucleotide substitutions, at positions 1097 (from G to C, designated 1097G > C) and 2303 (from C to A, 2303C > A) on the cDNA, resulting in amino acid

6.48 μg/mL and 0.182 mg/mL, respectively, and the ratio was 31.8 μmol uracil/g creatinine.

Immunoblot analysis of peripheral blood mononuclear cell cytosol. PBMC cytosol from the proband and her son and daughter were subjected to immunoblot analysis using a polyclonal antibody raised against human DPD to determine expression levels of DPD protein. As shown in Fig. 3, only a trace amount of DPD protein (105 kDa) was detected in the cytosol from the proband. This polyclonal antibody cross-reacted with an unknown protein band that was slightly larger than the DPD band and was present only in PBMC cytosol but absent in liver cytosol. Expression levels of DPD in cytosol from the son and daughter were comparable with that of the control cytosol.

Reverse transcriptase-PCR analysis of dihydropyrimidine dehydrogenase coding sequence. RT-PCR on total RNA isolated from PBMC of the proband and her son and daughter was done to analyze the full-length coding sequence of DPD. The coding region of DPD cDNA was amplified in three overlapping fragments with lengths of 1,157, 1,177, and 1,368 bp. Sequence analysis of these fragments showed the existence of two novel nucleotide substitutions, at positions 1097 (from G to C, designated 1097G > C) and 2303 (from C to A, 2303C > A) on the cDNA, resulting in amino acid

To determine whether the amino acid substitutions G366A and T768K in the DPD protein were responsible for very low PBMC-DPD activity of the proband, mutant types of DPD were expressed in E. coli in the His-tagged form and purified to homogeneity. DPD proteins, wild and mutant types (DPD-G366A, DPD-T768K, and DPD-G366A/T768K), were expressed in bacterial cytosolic fractions at levels similar to those examined by immunoblot analysis (data not shown). Each recombinant His-tagged DPD protein was purified as a homogeneous enzyme protein from the bacterial cytosol by Ni affinity and ADP-Sepharose affinity column chromatography. Final yield of each purified protein was ~450 to 600 μg from each 1 liter of culture medium. SDS-PAGE analysis indicated no difference in molecular mass (110 kDa) between the wild and three mutant types of proteins.

Enzyme activity of dihydropyrimidine dehydrogenase mutants. Enzymatic properties of the purified proteins of wild and mutant types of DPD were determined according to their 5-FU reducing activity in the presence of NADPH. Kinetic variables for the reaction indicated that DPD mutants containing the G366A substitution (DPD-G366A and DPD-G366A/T768K) had a markedly reduced affinity toward NADPH (49- and 77-fold increase in $K_m$ values), whereas this substitution did not influence the affinity to 5-FU (Table 4), resulting in a significant decrease in $V_{max}/K_m$ values for NADPH. With this mutation, $V_{max}$ values for 5-FU were decreased to ~0.5 of that for wild-type DPD. The T768K mutation neither influenced kinetic variables for the reduction of 5-FU nor affinity to NADPH.

Thermal stability of mutant dihydropyrimidine dehydrogenase proteins. To determine whether these mutations affect thermal stability of the enzyme proteins, 5-FU-reducing activity of wild DPD and DPD mutants was measured after preincubation for various periods at 45°C in the absence of NADPH and 5-FU (Fig. 5). Although thermostability of DPD-G366A was similar to that of wild DPD, DPD-T768K, and DPD-G366A/T768K, containing a common mutation at the position 768, lost their activity much faster than did wild DPD. After a 20-minute preincubation, the remaining activity of DPD-T768K and DPD-G366A/T768K was <5% of the initial

### Table 3. PBMC-DPD activity in 150 healthy Japanese volunteers

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>PBMC-DPD activity (nmol/min/mg protein)</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Max</th>
<th>Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>150</td>
<td>0.173 ± 0.055</td>
<td>0.166</td>
<td>0.371</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>75</td>
<td>0.181 ± 0.058</td>
<td>0.168</td>
<td>0.350</td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>75</td>
<td>0.165 ± 0.052</td>
<td>0.161</td>
<td>0.371</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>30</td>
<td>0.151 ± 0.030</td>
<td>0.150</td>
<td>0.228</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>30</td>
<td>0.166 ± 0.047</td>
<td>0.167</td>
<td>0.285</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>40-49</td>
<td>30</td>
<td>0.163 ± 0.042</td>
<td>0.157</td>
<td>0.263</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>50-64</td>
<td>30</td>
<td>0.172 ± 0.070</td>
<td>0.165</td>
<td>0.371</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>65-81</td>
<td>30</td>
<td>0.214 ± 0.060*</td>
<td>0.217</td>
<td>0.320</td>
<td>0.118</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from mean activities of other age groups ($P < 0.05$).
activity, whereas wild DPD and DPD-G366A still had ~20% of the initial activity. The presence of the cofactor NADPH (200 μmol/L) did not affect the stability of each DPD protein (data not shown).

Discussion

The present data are the first to be reported on the population distribution of PBMC-DPD activity in Japanese. Activity distribution among 150 healthy volunteers revealed no significant racial difference in the distribution pattern of activity between Japanese and Caucasian, Kenyan, Southwest Asian, and Korean populations. However, Sohn et al. (19) reported a significant difference between genders (men > women) in PBMC-DPD activity in a Korean population and higher mean activity than in reports on French and American populations. Our data on mean activity (0.173 nmol/min/mg protein) was close to that reported by Lu et al. (ref. 6; 0.189 nmol/min/mg protein) and Etienne et al. (ref. 16; 0.222 nmol/min/mg protein). Etienne et al. (16) also reported a statistically significant sex difference in PBMC-DPD activity (men > women) in 185 cancer patients, whereas Lu et al. (6) reported no significant sex difference in 124 healthy volunteers. Etienne et al. (16) suggested that this discrepancy was due to choice of the population itself and range of ages of subjects. Therefore, in designing the present study, we included equal numbers of men (n = 75) and women (n = 75) and included a wide range of ages. In the present study, the mean PBMC-DPD activity in women was slightly lower than in men, although without statistical significance. In addition, no significant sex difference was observed even if the comparison was done for each age group.

A weak but significant correlation (r = 0.346, P < 0.01) was observed between age and DPD activity (Fig. 2). When the comparison was made among age groups as reported by Lu et al. (6) and Etienne et al. (16), we found a statistically significant

Fig. 3. Immunoblot analysis of PBMC cytosol from the proband and her family. Each lane contains 10 μg protein of liver or PBMC cytosol. Cytosol was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection of DPD protein was done by the Enhanced Chemiluminescence Western Blotting Analysis System using rabbit anti-serum raised against human DPD. The molecular mass (105 kDa) of DPD protein was indicated by an arrow on the left side of the blot.

Fig. 4. Identification of 1097G>C and 2303C>A mutations by RT-PCR and genomic PCR. A, direct sequencing was done on amplified cDNA fragments by RT-PCR using total RNA isolated from PBMC of the proband. 1097G>C and 2303C>A mutations (arrows). The nucleotide positions of mutations are indicated on a diagram of the coding region of human DPD cDNA. Closed boxes represent putative binding sites for NADPH, FAD, pyrimidine, and (4Fe-4S) clusters. B, mutations (1097G>C and 2303C>A) identified on amplified exon fragments 10 and 19 by genomic PCR, respectively.
been identified in the human DPD gene (DPYD) (23), and at least 33 variant alleles have been identified in this gene. In addition, the proband and her husband were found to have at least one of the same mutations in their genome. The proband showed the existence of two novel point mutations. These mutations were strongly suggested to be non-synonymous because of a change in amino acid and thus are likely to influence enzyme activity. There is no synonymous mutation that correlated with reduced DPD activity (41).

The proband had very low PBMC-DPD activity (0.014 nmol/min/mg protein), which was below the lower limit of the 99% distribution range. Redetermination of PBMC-DPD activity from a blood sample collected on another day still showed very low activity (0.008 nmol/min/mg protein), indicating that the first result was not an artifact.

Sumi et al. (30) proposed that screening of the urinary uracil level can diagnose not only DPD deficiency but also dihydropyrimidinuria. Based on their analysis of uracil concentrations in urine samples from 1,133 people, they proposed a reference range of uracil concentration for the normal population of 25.1 to 99.8 μmol uracil/g creatinine. Kouwaki et al. (23) reported a high uracil/creatinine ratio (141.7 μmol uracil/g creatinine) in a patient with DPD deficiency. However, in the present study, urinary uracil concentration of the proband was 31.8 μmol uracil/g creatinine, which seemed to be within the reference range.

RT-PCR analysis of the full-length coding region of DPD in the proband showed the existence of two novel point mutations. These mutations were strongly suggested to be on one allele based on the inheritance of the genotype as determined by genomic sequence analysis of her children, which showed the same mutations in their genome. However, because of unavailability of a blood sample from her husband, we could not exclude the possibility that these mutations were divided between each allele and that the husband had at least one of the same mutations in his genome. In addition, the proband and her husband were unrelated.

Since Meinsma et al. (31) reported the molecular basis of DPD deficiency in a British family, at least 33 variant alleles have been identified in the human DPD gene (DPYD; refs. (32–39)). These mutant alleles contain single amino acid substitutions, nucleotide base deletions, or a donor site mutation resulting in exon skipping. Kouwaki et al. (23) reported three mutations in the DPYD gene in a Japanese cancer patient with decreased DPD activity accompanied by severe 5-FU toxicity. By a heterologous expression study, they showed that these mutations resulted in decreased or no activity of mutant DPD proteins. Yamaguchi et al. (40) reported frequencies and types of germ line mutations in the DPYD gene in 107 Japanese subjects consisting of cancer patients and healthy volunteers. They identified three novel mutations with amino acid substitutions other than the already-reported mutations. However, no further information on the DPD activity of these subjects who carry the novel mutations has been provided. Genotyping of 100 DPYD alleles from 50 Japanese subjects revealed that there was no mutation that correlated with reduced DPD activity (41).

Expression study of three types of mutant DPD proteins showed that the G366A mutation caused a markedly decreased affinity of the enzyme to the cofactor NADPH (Table 4). This mutation also influenced V_max to reduce the reaction velocity to ∼0.5. The glycine residue at position 366 is conserved among rat (42), mouse (43), porcine (25), bovine (44), and human (25) DPDs and is located at a close distance from the putative NADPH binding site (amino acid positions, 335-351) in the primary structure. According to the report on the crystal structure of pig DPD (45), Arg^{165} (next residue to Gly^{166}) was thought to possibly interact with the 2'-phosphate group of

Table 4. Kinetic parameters for the reduction of 5-FU by wild and mutant types of purified recombinant DPD

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>5-FU (μmol/L)</th>
<th>NADPH (μmol/L)</th>
<th>5-FU (μmol/min/mg protein)</th>
<th>NADPH (μmol/min/mg protein)</th>
<th>V_max/Km (μmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPD-wild</td>
<td>1.0</td>
<td>2.9</td>
<td>1,342.3</td>
<td>947.2</td>
<td>1,342.3</td>
</tr>
<tr>
<td>DPD-G366A</td>
<td>11</td>
<td>143.2</td>
<td>687.6</td>
<td>998.4</td>
<td>625.1</td>
</tr>
<tr>
<td>DPD-T768K</td>
<td>10</td>
<td>4.3</td>
<td>1,110.5</td>
<td>856.5</td>
<td>1,110.5</td>
</tr>
<tr>
<td>DPD-G366A/T768K</td>
<td>11</td>
<td>224.0</td>
<td>659.1</td>
<td>907.9</td>
<td>599.2</td>
</tr>
</tbody>
</table>

1 Reduction of 5-FU by wild and mutant types of purified DPD was done at various concentrations of 5-FU (range 0.5-200 μmol/L) in the presence of 200 μmol/L NADPH.

2 For determination of dependence on NADPH concentration, the cofactor was used at concentrations from 0.5 to 1000 μmol/L in the presence of 20 μmol/L 5-FU.

Fig. 5. Thermostability of wild and mutant types of recombinant human DPD. 5-FU-reducing activity of wild DPD and DPD mutants was determined after preincubation for various time periods at 45 °C in the absence of NADPH and 5-FU.
Gly366 residue is located at a close distance (~10 Å) to the NADPH molecule, which was co-crystallized with pig DPD.

Thermal stability experiments showed that the T768K mutation caused a marked decrease in thermal stability of the mutant DPD (Fig. 5), indicating that T768 could play a role in the structural integrity of the DPD protein. However, the G366A substitution did not affect thermal stability of DPD protein. These data suggested that the T768K mutation also might cause DPD deficiency because of protein instability in living cells. The effects of a single amino acid substitution on thermal stability of enzyme proteins, such as human superoxide dismutase (46) and dog catalase (47), and on a decrease in susceptibility to protein degradation have been reported. It was reported that a mutation (A4V) of superoxide dismutase led to thermal instability of the enzyme and was linked to the familial form of amyotrophic lateral sclerosis (46). A mutant type of dog catalase was found in atacalasemic dog liver and was shown to be rapidly degraded in COS-1 cells (47). In the present study, the result of immunoblotting showed markedly reduced expression of DPD protein in PBMC cytosol of the proband. This might be due to instability of DPD protein and cause very low DPD activity and expression in PBMC.

An unexplained discrepancy was that the proband’s children had only weak DPD activity (~50% of mean activity), although they had the same genotype as the proband. We could not determine the reason for the very low expression level of DPD protein in the proband whereas her children expressed DPD protein at almost a normal level. Therefore, further investigation is required to determine the mechanism of the very low DPD expression in the proband, including sequence analysis of the 5′-flanking region of the DPYD gene. Collie-Duguid et al. (48) reported human DPYD gene promoter analysis and suggested that a polymorphism at a putative AP-3 site and γ-IRE in the 5′-flanking region of the gene may cause reduced DPD activity and 5-FU intolerance. A report on genotyping of cancer patients with reduced or normal DPD activity from the same laboratory showed that only 17% of those patients had a molecular basis for their deficient phenotype, which emphasized the complex nature of the molecular mechanisms controlling polymorphic DPD activity in vivo (33).

In patients with very low or deficient DPD activity, 5-FU chemotherapy is associated with severe or life-threatening toxicity (15, 25, 49). It was also reported that 5-FU clearance was markedly reduced in a patient who was partially DPD deficient due to heterozygosity for a mutant allele of the DPYD gene (35). Therefore, 5-FU or its prodrugs should not be given to patients who are DPD-deficient or who have very low DPD activity in liver or in PBMC; otherwise, they will suffer from severe toxicity or die from markedly elevated tissue 5-FU levels (15, 38, 50).

In the present study, we found one healthy volunteer (0.7% of the population) with very low PBMC-DPD activity due to heterozygosity for a mutant allele of the DPYD gene in a population of 150 Japanese, whereas it has been estimated that the frequency of very low DPD activity was ~0.1% in the normal population. Further screening of PBMC-DPD activity in a larger population is required to determine accurately the frequency of DPD deficiency in Japanese.

References

24. Koutsaki M, Harajama N, Suri S, et al. Identification of NADPH as a neutralizing base. These findings suggest that the G366A mutation may cause a conformational change of the environment around the NADPH binding site that leads to reduced affinity. In general, the glycine residue is much less sterically hindered than other amino acids and often occupies positions where a polypeptide backbone makes a sharp turn in the three-dimensional structure of protein, and the substitution of glycine for other amino acids disrupts these structures. Based on the reported crystal structure of pig DPD, the Gly366 residue was located at a close distance (~10 Å) to the NADPH molecule, which was co-crystallized with pig DPD.
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