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Research Article

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Molecular Basis of the Human Dihydropyrimidine Dehydrogenase Deficiency and 5-Fluorouracil Toxicity

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Abstract

Dihydropyrimidine dehydrogenase (DPD) deficiency constitutes an inborn error in pyrimidine metabolism associated with thymine-uraciluria in pediatric patients and an increased risk of toxicity in cancer patients receiving 5-fluorouracil (5-FU) treatment. The molecular basis for DPD deficiency in a British family having a cancer patient that exhibited grade IV toxicity 10 d after 5-FU treatment was analyzed. A 165-bp deletion spanning a complete exon of the *DPYD* gene was found in some members of the pedigree having low DPD catalytic activity. Direct sequencing of lymphocyte DNA from these subjects revealed the presence of a G to A point mutation at the 5′-splicing site consensus sequence (GT to AT) that leads to skipping of the entire exon preceding the mutation during pre-RNA transcription and processing. A PCR-based diagnostic method was developed to determine that the mutation is found in Caucasian and Asian populations. This mutation was also detected in a Dutch patient with thymine-uraciluria and completely lacking DPD activity. A genotyping test for the G to A splicing point mutation could be useful in predicting cancer patients prone to toxicity upon administration of potentially toxic 5-FU and for genetic screening of heterozygous carriers and homozygous deficient subjects. (*J. Clin. Invest.* 1996. 98:610–615.) Key words: DPD deficiency • 5-FU toxicity • population genotyping • exon skipping

Introduction

The human dihydropyrimidine dehydrogenase gene (*DPYD*)[1] encodes dihydropyrimidine dehydrogenase (DPD, E.C. 1.3.1.2) which is the initial and rate-limiting enzyme in the three-step metabolic pathway leading to the catabolism of the pyrimidine bases uracil and thymine (1). This is the only metabolic pathway in the biosynthesis of β-alanine in mammals. DPD is also the key enzyme that degrades the structurally related pyrimidine antimetabolite 5-fluorouracil (5-FU), a common anticancer drug used in the treatment of colon, breast, head, neck, and ovary tumors (2). Since 70–80% of the administered 5-FU is degraded in vivo by DPD to fluorinated β-alanine (3), the level of DPD activity is a major determinant in the toxicity of 5-FU.

DPD deficiency was first described in pediatric patients exhibiting thymine-uraciluria, which is associated with a variety of symptoms including convulsive disorders (epilepsy), microcephaly, and mental retardation (4–7). Since the first report of an adult cancer patient that under 5-FU chemotherapy developed severe toxicity and had low DPD activity (8), additional cases of DPD deficiency have been reported (9–11). In family studies using both pediatric and cancer patients, an autosomic recessive pattern of inheritance for DPD deficiency was suggested based on measurements of catalytic activity in lymphocytes (9, 12). A frequency as high as 3% of putative heterozygotes for DPD deficiency was also estimated based on catalytic activity levels in population studies (13, 14). Using the Hardy-Weinberg equilibrium, this frequency of heterozygotes allows the estimation of up to 1/1,000 homozygotes for mutant *DPYD* alleles.

The identification and characterization of the human DPD cDNA (15) made possible the identification and molecular analysis of mutations that affect DPD expression and catalytic activity. A pediatric patient with complete lack of DPD catalytic activity and his family were examined previously and found to possess an mRNA with a deletion that is incapable of producing a functional enzyme (12). However, the mechanism responsible for the deletion could not be resolved. In this report, we studied an unrelated British family having a cancer patient with partial DPD deficiency and severe toxicity after 5-FU treatment. The same deletion at the mRNA level was found. By cloning the human *DPYD* gene, and by determination of its intron-exon boundaries, a PCR-based genotyping test was developed that allowed the molecular analysis of the deletion. We found that a G to A point mutation within the 5′-splicing site (GT to AT), which appears to cause exon skipping, leads to an inactive *DPYD* allele. A diagnostic assay was developed to determine allele frequency in the normal population and the results indicated that this mutation may be a common mechanism for DPD deficiency and exaggerated 5-FU toxicity in cancer patients.

Methods

Family pedigree. Blood samples from a British family were collected after a family member displayed excessive 5-FU toxicity during chemotherapy. This family is unrelated to any family with DPD defi-

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1. *Abbreviations used in this paper:* DPD, dihydropyrimidine dehydrogenase; *DPYD*, dihydropyrimidine dehydrogenase gene; 5-FU, 5-fluorouracil.

ciency that has been reported previously. To screen for the presence of this mutation in the normal population, genomic DNA was collected from healthy subjects representing the following ethnic groups: Caucasians (English and Finnish), Asians (Japanese and Taiwanese), and African-Americans. The proband (subject I1), was a 65-yr-old man who presented grade IV pancytopenia and mucositis 10 d after his first course of 5-FU as part of adjuvant therapy for the treatment of colorectal cancer and had catalytic activity which was 24% of the mean population.

DPD catalytic activity. DPD catalytic activity for the family under study was determined from peripheral blood mononuclear cells using a previously described HPLC method (16). In brief, peripheral blood mononuclear cells were purified from the subjects' blood in a density gradient using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and incubated for 90 min with [^{14}C]5-FU. The production of 5-FU metabolites was then quantified by HPLC analysis with radiodetection.

Isolation of RNA and reverse transcriptase (RT)-PCR. Total RNA was isolated from peripheral blood mononuclear cells by the guanidinium thiocyanate-phenol-chloroform method (17). The RNA solutions were dissolved in DEPC-treated water and stored at −80°C until use. RT-PCR was performed as described (12) and the products were analyzed by electrophoresis in 0.8% agarose gels and visualized by staining with ethidium bromide.

Cloning and characterization of the genomic fragment encoding the deleted exon. The DPD cDNA was used as a probe to isolate a P1 clone containing ~ 100 kbp of the human *DPYD* gene (PAC 5945) from a high density PAC human genomic library (Genome Systems, St. Louis, MO). Southern blotting was used to confirm that the P1 clone contained the deleted exon using a probe located within the deleted fragment. This probe was synthesized from the DPD cDNA by using the primers: DPD15F (forward): 5′ TTGTGACAAATGTTTCCC 3′ and DPD15R (reverse): 5′ AGTCAGCCTTTAGTTCAGTGACAC 3′ to specifically amplify the putative exon. PCR conditions were as indicated below but extension was carried out at 72°C for 1 min. This PCR fragment was purified using a Wizard PCR purification kit (Promega, Madison, WI), labeled with [α-^{32}P]dCTP, and hybridized with the clone PAC 5945. DNA was purified from this genomic clone using Qiagen columns (Qiagen, Chatsworth, CA), and the 5′ and 3′ ends of the deleted exon and adjacent intronic regions were sequenced by chromosome walking from within the deleted exon using dideoxy terminator chemistry and an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA). The intronic sequences obtained allowed the selection of appropriate primers (DPDdelF1 and DPDdelR1) to amplify from genomic DNA the complete exon and immediate flanking intronic sequences. All primers used in this study were synthesized with a 394 DNA&RNA synthesizer (Applied Biosystems).

Genotyping of the mutant DPYD allele. A 409-bp PCR genomic fragment corresponding to the deleted exon (from G1822 to C1986 in reference 15) plus the flanking intronic sequences containing the AG and GT splicing consensus sequences was amplified from human genomic DNA using the primers DPDdelF1 (forward) 5′ TGCAAATATGTGAGGAGGGACC 3′ and DPDdelR1 (reverse) 5′ CAGCAAAGCAACTGGCAGATTC 3′. PCR amplification was carried out in a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 2.5 U of *Taq* Polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), and 500 ng of genomic DNA template for 31 cycles by denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extending at 72°C for 2 min. Subjects identified as wild-type, heterozygous, or homozygous for the splicing mutation could be distinguished by digesting the PCR product with the restriction endonuclease Mae II (Boehringer Mannheim, Indianapolis, IN) and electrophoresis in 1% regular, 3% NuSieve agarose gels (FMC Bioproducts, Rockland, ME). The genotypes obtained were verified by sequencing the 409-bp PCR product. The sources of the genomic DNA samples for the different ethnic groups correspond to those described previously (18).

Table I. Relationship between Phenotype and Genotype for the DPD Catalytic Activity and the Presence of the 5′-Splicing Mutation in the British Pedigree Analyzed

Family member	DPD activity*	Genotype‡
I1	37	+/−
II1	88	+/+
II2	60	+/−
II3	56	+/−
II4	65	+/−
II5	ND	ND
II6	54	+/−
II7	90	+/−
II8	34	+/+
II9	43	+/−
III1	85	+/+
III2	93	+/+
III3	ND	ND
III4	93	+/+
III5	ND	ND
III6	56	+/−
III7	108	+/+
III8	48	+/−
III9	87	+/−

*DPD activity is expressed as pmol/min/mg protein. The mean in the normal population corresponds to 155 pmol/min/mg protein as determined using the HPLC procedure indicated in Methods. ‡Genotype for the GT to AT splice mutation: +/+: wild-type; +/−: heterozygote; −/−: homozygote; ND, not determined. The calculated mean for wild-type (+/+) subjects is 87±18 pmol/min/mg protein, whereas for heterozygote (+/−) subjects it is 58±19 pmol/min/mg protein. The difference in DPD activity between the +/+ and the +/− subjects in this pedigree is statistically significant ($P < 0.01$).

Results

DPD catalytic activity. To determine DPD catalytic activities in the proband and family members, lymphocytes were isolated and subjected to analysis by HPLC (16). Activities for this British family ranged between 22 and 70% of the mean DPD activity (155 pmol/min/mg protein) determined in our laboratory in the normal population using the HPLC protocol indicated in Methods (Table I and Fig. 1). It should be noted that all the subjects in the pedigree presented a relatively low DPD catalytic activity that is below 70% of the mean in the normal population.

RT-PCR analysis. RT-PCR was performed on total RNA isolated from peripheral blood mononuclear cells corresponding to each of the members of the pedigree shown in Fig. 1. The full-length coding region of the DPD cDNA was amplified in three fragments of 1.5 kbp, 906 bp, and 919 bp. The 1.5-kbp and 906-bp PCR fragments were detected in all the members of the family and correspond to the normal mRNA as expected from the cDNA sequence (results not shown). As shown in Fig. 2 *A*, the 741-bp band corresponds to the deleted exon and was heterozygous with respect to the wild-type 906-bp fragment. Sequencing of this 741-bp PCR fragment confirmed that a 165-bp deletion has occurred that precisely corresponds to that reported previously in a Dutch family (12). No

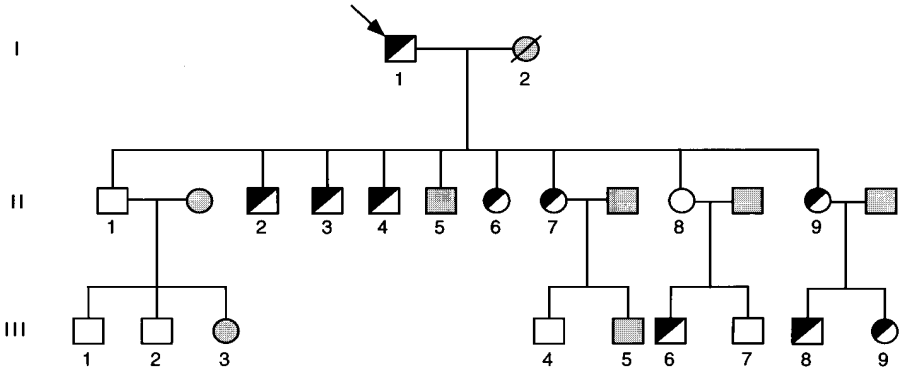

Figure 1. Schematic representation of the British pedigree used in this study. The cancer patient exhibiting 5-FU toxicity is indicated by an arrow. The different generations of the family are denominated I, II, and III. Each member of every generation is marked by Arabic numerals. Half filled symbols stand for heterozygotes (+/−) and open symbols for wild-type (+/+) subjects for the *DPYD* alleles. Dotted symbols correspond to those subjects not determined.

homozygous mutant subjects were found in this pedigree. The generation of the mutant mRNA is illustrated in Fig. 2 *B*. Faulty splicing is the most likely mechanism for the generation of the shorter mRNA, which due to the absence of 55 amino acids would be unable to be translated into an intact DPD enzyme.

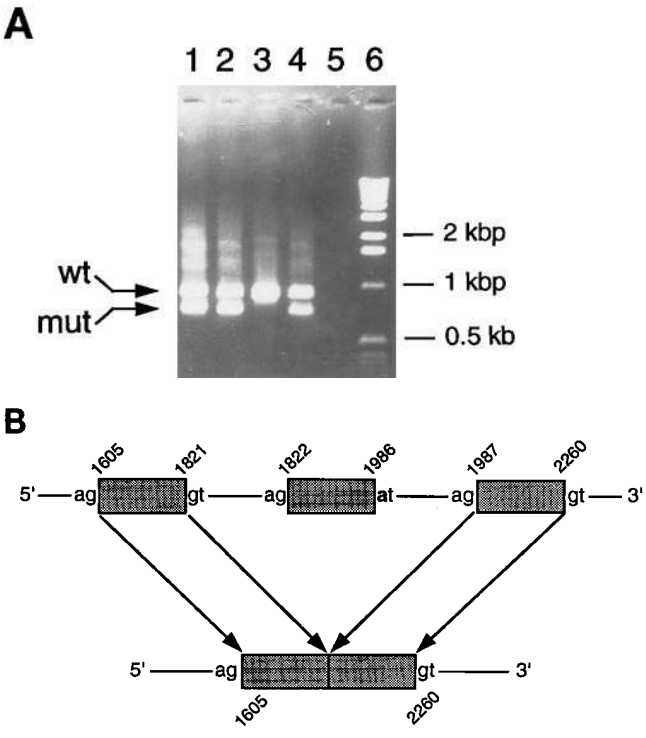

Figure 2. Proposed mechanism for the deletion present in the DPD cDNA of the pedigree under study. (*A*) Agarose gel electrophoresis for representative subjects of the pedigree showing the detection of the normal (*wt*) and mutant (*mut*) cDNA fragments after RT-PCR amplification from lymphocyte total RNA. The mRNA bands correspond to 909 (*wt*) and 741 bp (*mut*). Representative heterozygote subjects are shown in lanes *1*, *2*, and *4*; lane *3* corresponds to a wild-type subject. Lane *5* is a negative control in the absence of RNA template for the RT-PCR reaction and lane *6* contains 1 kbp DNA ladder as size markers (GIBCO-BRL, Gaithersburg, MD). (*B*) Scheme representing the generation of the mutant DPD cDNA after a fragment of 165 bp from the *DPYD* gene corresponding to a complete exon is removed by faulty splicing. Exons are indicated by dotted boxes, introns are represented by solid lines.

Genomic DNA sequence analysis of the mutant DPYD allele. A clone containing the deleted exon was isolated in a recombinant P1 phage. Sequence of the intronic regions flanking the deleted exon enabled the development of a PCR reaction to sequence the exon across the 5′ and 3′ splicing sites. Sequence of the 409-bp PCR genomic fragment clearly indicated the presence of a G to A point mutation (GT to AT) at the 5′ donor splice consensus sequence in the British pedigree in subjects I1, II2, II3, II4, II6, II7, II9, III6, III8, and III9 (+/− in Fig. 3). This splicing mutation was not found in subjects II1, II8, III1, III2, III4, and III7 (+/+ in Fig. 3). The subject previously reported to have the 165-bp deletion present in both alleles and his heterozygote brother (subjects 1 and 2 in reference 12) were also analyzed and found to possess the splicing mutation present in both alleles for subject 1 (indicated as −/− in Fig. 3) and in one allele for subject 2. The G to A mutation correlates precisely with the corresponding RT-PCR results for the deletion of the entire exon, indicating that the GT to AT mutation in the 5′ splicing consensus sequence of the *DPYD* gene leads to skipping of the entire preceding exon. The genotypes and catalytic activities for this 5′-splicing mutation in the pedigree under study are summarized in Table I. All the members of this family presented a level of DPD activity below the mean in the population, wild-type subjects had a mean activity of 87±18 pmol/min/mg protein whereas heterozygote subjects had a mean of 58±19 pmol/min/mg protein. Statistical analysis (*t* test) indicated that these two groups are significantly different from each other with respect to catalytic activity ($P < 0.01$) (Table I).

Genotyping test for the mutant DPYD allele and population screening. The 409-bp genomic PCR fragment containing the deleted exon has a restriction site for the endonuclease Mae II (A-CGT) which produces two fragments of 278 and 131 bp from the wild-type allele. This site is eliminated when a G to A mutation occurs at the 5′-splicing site (A-CAT). A diagrammatic representation of the strategy developed for PCR amplification of the 409-bp fragment and the restriction enzyme pattern expected with Mae II is shown in Fig. 4 *A*. The results obtained for representative members of the pedigree under study are shown in Fig. 4 *B*. The reliability of the genotyping assay was confirmed by sequencing the 409-bp PCR product in these samples (Fig. 3). By analyzing normal subjects within different ethnic groups, we found that this mutant allele is present in the Finnish (2.2% out of 90 alleles analyzed) and Taiwanese (2.7% out of 72 alleles) populations where heterozygotes were detected. However, within the British samples tested (60 alleles), no mutant *DPYD* alleles were found with

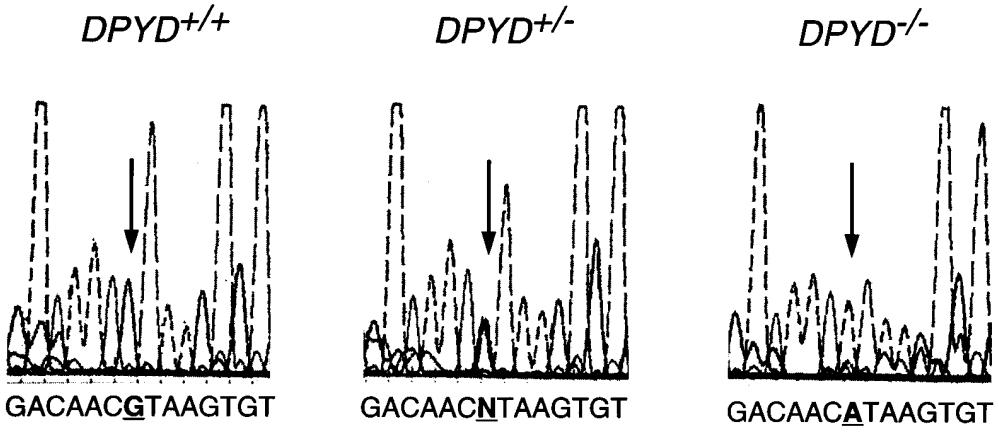

Figure 3. Direct sequencing of the 409-bp PCR product amplified from genomic DNA using the primers DPDdelF1 and DPDdelR1 (see Methods). The nucleotide where the mutation takes place and that causes faulty splicing is indicated in each panel by an arrow. The genotype of each subject is indicated at the top. In the heterozygous subject (+/−), the base N indicates the comigration of the wild-type and mutant alleles at that specific nucleotide position. The homozygous mutant subject (−/−) corresponded to that previously reported (12).

the exception of the pedigree shown in Fig. 1. No mutant alleles were found in 35 Japanese (70 alleles) or 20 African-American (40 alleles) subjects. All heterozygous subjects found by PCR-restriction enzyme analysis were also confirmed by direct DNA sequencing of the PCR product.

Discussion

A GT to AT mutation in the 5′-splicing consensus sequence in the human *DPYD* gene was uncovered that leads to skipping of the entire exon preceding the mutation. In the 5′-splicing consensus sequence, the dinucleotide GT is the best conserved among mammals, and mutations affecting this sequence were reported to cause the inactivation of several genes associated with human diseases including *phenylalanine hydroxylase* (19), *procollagen COL3A1* (20), and *hypoxanthine guanine phosphoribosil-transferase* (21). In these genes, a G to A mutation in the GT splicing recognition sequence consistently results in skipping of the entire exon preceding the mutation, supporting the current theory of exon definition for higher eukaryotes in which the splicing sites are recognized as exonic pairs as opposed to the mechanism proposed for lower eukaryotes in which the splicing sites are recognized as intronic pairs (22, 23).

The generation of a mutant allele by a splicing site mutation was confirmed by analyzing the DNA of a Dutch pedigree having a subject determined by RT-PCR of fibroblast RNA to be homozygous for the same deletion. The skipped exon resulted in a deletion of 55 amino acid residues in the primary sequence of the DPD protein (12, 15). As these data indicate, the presence of the splicing mutation in both human alleles leads to a complete deficiency in DPD protein expression and catalytic activity. Additionally, a precise correlation between presence of the splicing mutation and deletion of the preceding exon, as assessed by RT-PCR, was observed in all the subjects of the Dutch and British pedigrees where the splicing mutation was also found. Subjects identified as having two wild-type alleles, with respect to this mutation, had normal DPD mRNAs. A pediatric patient of Pakistani origin exhibiting thymineuraciluria and having undetectable DPD activity was also homozygous for the 5′-splicing mutation (our unpublished observations).

It was suggested that the level of DPD activity observed in cancer patients having 5-FU toxicity is within the same range

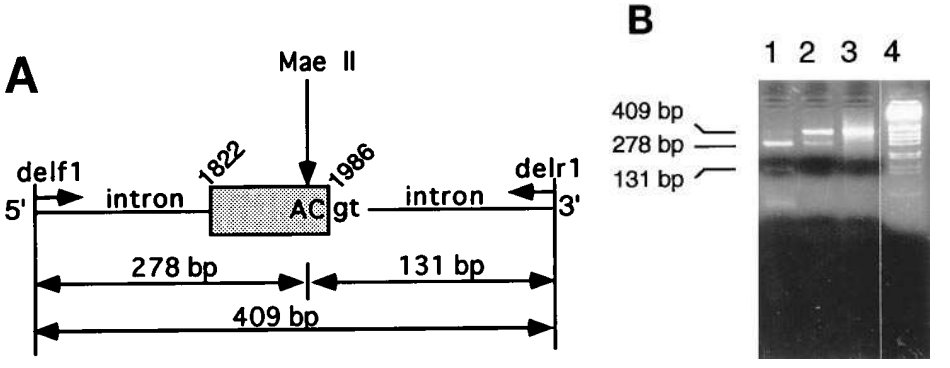

Figure 4. PCR genotyping assay to detect the GT to AT mutation causing altered splicing in the *DPYD* gene. (*A*) Schematic representation of the strategy developed to amplify by PCR the deleted exon and the corresponding splicing consensus sequences. The nucleotide position of the exon in the cDNA is indicated. DelF1 and DelR1 stand for the primers DPDdelF1 and DPDdelR1, respectively (see Methods). The restriction endonuclease Mae II produces fragments of 278 and 131 bp in the wild-type allele. A point mutation at the donor splicing consensus sequence (GT to AT) destroys this restriction site and therefore the mutant allele remains undigested (409 bp). Exons are indicated by dotted boxes, introns are represented by solid lines. (*B*) Agarose gel electrophoresis for the detection of mutant *DPYD* alleles. The wild-type *DPYD* allele gives after Mae II restriction fragments of 278 and 131 bp, whereas the mutant allele gives a unique 409-bp band. The sizes of the fragments generated for the different genotypes are indicated. These results were confirmed by sequencing the 409-bp PCR fragment (see Fig. 3). Lanes *1*, *2*, and *3* correspond to wild-type, heterozygote, and homozygote mutant subjects, respectively. Lane *4* contains 1-kbp DNA ladder as size markers (GIBCO-BRL).

of that observed in heterozygous members from families having DPD homozygous mutant subjects (1, 24). The results presented here also provide evidence that 50% of the normal level of DPD activity in cancer patients is sufficient to trigger development of severe degrees of toxicity with 5-FU. These data clearly establish that inactivation of one *DPYD* allele results in a decrease in DPD expression large enough to cause 5-FU toxicity, thus providing a possible genetic basis for this toxicity. These data indicate that the mutation we observed results in a functionally inactive enzyme and that we have not amplified and analyzed a pseudogene. Additionally, we have no evidence suggesting the existence of a pseudogene from our genomic cloning studies.

In a limited screening study, the splicing mutation was found in heterozygous state in $\sim$ 4% of the Finnish and in $\sim$ 5% of the Taiwanese subjects analyzed. No homozygote mutants were detected in any other ethnic group analyzed in this study and no mutant alleles were found in random British, Japanese, or African-American populations. However, due to the small number of samples available from these latter populations, it remains a possibility that the mutant allele will be detected at some frequency after a larger population is studied. From population analysis of DPD catalytic activity (16, 25, 26), it was estimated that $\sim$ 3% of the normal population could be heterozygotes for mutant *DPYD* alleles, and using the Hardy-Weinberg equilibrium, up to 1 in every 1,000 births in the general population could be homozygous for *DPYD* mutations. Thus, the allele having the splicing mutation could be present in Taiwanese and Finnish populations at the same or even higher frequency than that estimated from catalytic activity–based populations studies. A genotyping method based on the G to A splicing site mutation could be useful for the screening of cancer patients who are to undergo 5-FU chemotherapy due to the apparent wide-spread occurrence of this mutant allele in Caucasians and Asians.

A significant level of variability of DPD activity within the normal population has been reported (16, 25, 27). We have amplified by PCR and directly sequenced 17 out of the 22 exons present in the *DPYD* gene (results not shown) from all the subjects of the pedigree analyzed here. Three different point mutations that produce nonconservative amino acid changes were identified in subjects that are wild-type for the splicing mutation reported in this study (unpublished experiments). It is a possibility that these amino acid changes affect their level of catalytic activity. The effect of these mutations on the catalytic activity of the British pedigree analyzed here and their frequency in the normal population are currently under study. The identification of different mutations in the normal population implies not only the existence of different *DPYD* alleles but also that different combinations of them could account for the wide variability of DPD activity observed with respect to the mean in the population. Alternatively, the influence of such factors as diet, pyrimidine homeostasis, and/or other regulatory processes is also considered to contribute to the large degree of variability of DPD activity in the normal population.

Since 5-FU is one of the most commonly prescribed chemotherapeutic drugs in cancer treatment, and the mutant *DPYD* allele reported here appears to be homogenized among different ethnic groups, the screening of cancer patients a priori could be a feasible approach for avoiding 5-FU toxic effects. It can also be used for the determination of mutation carriers and for prenatal diagnosis of DPD deficiency.

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