Cancer Chemother Pharmacol (2004) 53: 445–451
DOI 10.1007/s00280-003-0749-y

ORIGINAL ARTICLE

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Dihydropyrimidine dehydrogenase pharmacogenetics in the Taiwanese population

Received: 25 March 2003 / Accepted: 26 November 2003 / Published online: 17 January 2004

Abstract *Background/purpose*: 5-Fluorouracil (5-FU) remains the most frequently used chemotherapy agent in various human cancers. Over 80% of the 5-FU administered is metabolized by dihydropyrimidine dehydrogenase (DPD) in the liver. However, mutations in the DPD gene have been found to be associated with low DPD activity causing severe complications. The aim of this study was to determine the frequency of 11 known mutations in Taiwanese subjects and the relationship between mutation and DPD level. *Methods*: Samples from a total of 300 subjects were investigated in this study. The PCR-RFLP method was used to identify 11 mutations of the DPYD gene, including 62G > A, 74A > G, 85T > C (DPYD*9A), 812delT, 1003G > T, 1156G > T, 1627A > G (DPYD*5), 1714C > G, 1897delC (DPYD*3), 2194G > A (DPYD*6), and IVS14 + 1G > A (DPYD*2A). DPD protein levels were determined using a DPD ELISA kit. *Results*: Four mutations, including 74A > G, 85T > C (DPYD*9A), 1627A > G (DPYD*5), and 2194G > A (DPYD*6), were found in our 300 samples. The following mutations were not detected: 62G > A, 812delT, 1003G > T, 1156G > T, 1714C > G, 1897delC (DPYD*3), and IVS14 + 1G > A (DPYD*2A). The phenotype analysis by DPD protein level indicated that the 1627A > G (DPYD*5) mutation was not associated with the DPD protein level and might be a polymorphism in the DPD gene. The DPD level was also not correlated with gender. *Conclusion*: No significant correlations between these 11 mutations and DPD protein level were found indicating that examination of these mutations is insufficient to provide a high-value prediction of the 5-FU pharmacogenetic syndrome in Taiwanese. Genotype and phenotype analysis indicated the 1627A > G (DPYD*5) mutation to be a polymorphism.

Keywords Dihydropyrimidine dehydrogenase · DPD · DPYD gene · 5-Fluorouracil · 5-FU

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Introduction

The antimetabolite, 5-fluorouracil (5-FU), even though it was first synthesized over four decades ago, remains one of the most frequently used chemotherapy drugs against a wide spectrum of solid tumors, including carcinomas of the breast, gastrointestinal tract, head and neck, and ovary [3]. The cytotoxic effect of 5-FU depends on the anabolic pathway of the drug to cytotoxic nucleotides, which can then produce cytotoxicity through inhibition of thymidylate synthase activity and/or incorporation into RNA and DNA [3, 14, 27, 31]. Although the cytotoxic effects and probably the host toxicity depend on the anabolic pathways, the catabolic route is involved in the conversion of 80–90% of administered 5-FU into biologically inactive metabolites [5, 13]. The initial and rate-limiting enzyme of the pyrimidine catabolic pathway is dihydropyrimidine dehydrogenase (DPD) (EC 1.3.1.2), which is also the key enzyme in the degradation of the structurally related pyrimidine antimetabolite, 5-FU [23, 35, 37]. Increased levels of DPD activity corresponding to catabolism of 5-FU could result in 5-FU resistance and decreased levels of DPD activity and the consequent predominance of the anabolic pathway could result in an increase in the available drug. So DPD has a valuable role not only as a predictor of toxicity associated with 5-FU use, but also in the prediction of response to 5-FU therapy [1, 14, 23].

The association between 5-FU toxicity and DPD activity is best illustrated in patients with DPD

deficiency. After 5-FU treatment, patients develop profound toxicity including mucositis, granulocytopenia and neuropathy, and may even die [6, 10, 11, 12, 33]. Even topical 5-FU may be toxic: severe multiorgan toxicity in a DPD-deficient patient treated topically with 5-FU has been described [17]. In children, DPD deficiency is also accompanied by variable clinical presentations including neurological disorders, growth retardation, and dysmorphism [35]. This evidence indicates that patients with severe or complete DPD deficiency have a high risk of inborn diseases and of showing toxicities associated with the use of 5-FU. Although DPD activity is important in 5-FU therapy, the complicated method of assaying the enzyme has hindered its clinical usage in most treatment centers [16]. Many studies have also shown that the effects of DPD on 5-FU metabolism can be considered a pharmacogenetic syndrome because molecular defects in the DPD gene (DPYD) have been shown to result in profound suppression of DPD activity [7, 19, 20, 21, 22, 26]. This has led to several studies suggesting that the identification of the molecular defect may be necessary before 5-FU treatment [10, 28, 36, 39].

To date, more than 30 mutations in the DPYD gene have been reported. The G to A transition at the splice site which results in skipping of exon 14 (IVS14 + 1-G > A) (DPYD*2A) which results in truncated protein is thought to be the most common mutation associated with low DPD activity in Europeans [1, 14, 17, 21, 22, 23, 24, 28, 29, 35, 36, 39]. Other mutations, including deletion of the cytosine at position 1897 (1897delC) (DPYD*3), which causes premature termination of the protein at codon 633, and a point mutation at position 1627 (1627A > G) (DPYD*5) have also been reported [21, 23, 29, 35, 38]. In addition to the above mutations, novel mutations have also been found in Asian populations. Point mutations at positions 62 (62G > A), 1003 (1003G > T), and 1156 (1156G > T) in codon 386 which cause premature termination have been reported by Kouwaki et al. in a Japanese patient with 5-FU toxicity [19]. Yamaguchi et al. have added three novel mutations found in 107 Japanese individuals: 74A > G, 812delT and 1714C > G [40]. Although the data are limited, some differences in frequency between ethnic groups have been reported but there are few data derived from the Taiwanese population [29, 38, 39].

In this study, a total of 11 known Asian mutations of the DPYD gene, including 62G > A, 74A > G, 85T > C (DPYD*9A), 812delT, 1003G > T, 1156G > T, 1627A > G (DPYD*5), 1714C > G, 1897delC (DPYD*3), 2194G > A (DPYD*6), and IVS14 + 1G > A (DPYD*2A), were analyzed by the PCR-RFLP method in a Taiwanese population to determine whether there are mutations among the Taiwanese population and the mutant frequency. DPD protein levels were determined by an ELISA method and the relationship between genotype and DPD protein level was analyzed. In this way, we hoped to determine the frequency of these 11 known Asian mutations in the Taiwanese population and the relationship between genotype and DPD protein level. The data were also compared with those in other reports to determine whether there are differences between the Taiwanese population and other ethnic groups.

Subjects, materials and methods

Subjects

The study was supervised by the Kaohsiung Medical University IRB with approval number KMU-IRB:91-026. A total of 300 samples were evaluated in the study. Among them, 220 samples were stored DNA from our laboratory and 80 samples were from healthy volunteers who had provided informed consent. Blood was collected from a peripheral vein into a heparinized tube between 8 and 10 o'clock in the morning to avoid circadian effects [11, 23]. Mononuclear cells were isolated from whole blood by the Ficoll-Hypaque method. For DPD genotype analysis, cells were lysed by cell lysis buffer and the protein was removed in 10 *M* NH_4OAc solution. DNA was then precipitated with isopropanol, resuspended in 0.1% DEPC H_2O after washing in alcohol and stored at −20°C. For DPD protein analysis, mononuclear cells from volunteers were washed twice in Tris-buffered saline (TBS) and cell pellets were resuspended in 500 μl TBS. The cells were then lysed in a sonifier (three 5-s pulses on ice). After spinning down at high speed, the supernatant was collected carefully and stored at −80°C before DPD analysis which was carried out within 14 days of blood collection to avoid changes in the DPD level.

DPD genotype analysis

For mutation detection, the PCR-RFLP method was used. Primer sets and restrictive enzymes were designed according to the DPD DNA sequence and the mutant alleles (Table 1). PCR was carried out in a mixture of 10 m*M* Tris-HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, 0.2 m*M* of each dNTP, 2.5 U Taq polymerase, 100 ng genomic DNA template, and 100 ng of the primers listed in Table 1. The reaction was performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 35 cycles. The PCR products were digested by respective restrictive enzymes under the appropriate conditions. The resulting fragments were separated by 3% gel electrophoresis and visualized by ethidium bromide staining.

Sequence

To identify the mutations, the PCR products were subcloned with a DNA sequencing kit (Applied Biosystems, Foster City, Calif.) for a PCR reaction of 25 cycles. The cDNA clone was sequenced using an autosequencer (Applied Biosystems 310 genetic analyzer).

DPD ELISA method

Mononuclear cells from 72 healthy volunteers were prepared within 2 weeks of obtaining the samples for DPD protein determination. The DPD protein was determined by an ELISA method (catalogue no. 2207184; Roche, Basel, Switzerland) in duplicate for each sample [25]. In brief, the samples were added to wells which had previously been coated with monoclonal anti-DPD antibody (clone 4B9). After incubation at 37°C for 2 h at 300 rpm, the samples were washed three times with washing buffer. Then the samples were incubated again with another anti-DPD monoclonal antibody (clone 3A5) which conjugated with peroxidase at 25°C for 1 h at 300 rpm. After washing the samples three times again, ABTS

Table 1 Sequences of primer sets and restriction enzymes used for PCR-RFLP and the length of PCR products

Mutation	Primer	Restriction enzyme	Length of product (bp)	
			Wild	Mutant
62G > A	5′-TTTAGAGTATCCTGGCTTTA 3′-TGGTACTTACAAAGCAGTTC	Taq 1	100, 26	126
74A > G	5′-TGAGAGAGACCGTGTCTCA 3′-TGGCCGAAGTGGAGC	Nla III	139, 26	165
85T > C (DPYD*9A)	5′-TGAGAGAGACCGTGTCTCA 3′-TGGCCGAAGTGGAGC	Hha I	165	150, 15
812delT	5′-TGAATGAAATGACTCTTAGCA 3′-GGATATTGCTAGGAAATAAAA	Tag I	126	103, 23
1003G > T	5′-TGTCATGCAGAAATGGTTTC 3′-TCCAGCTCCAAGTACAATCG	Bst UI	98, 23	121
1156G > T	5′-TAGATGGAACTTGCTAAGGA 3′-GAACTGAACCAAAGGCACTG	Apo I	168, 31	199
1627A > G (DPYD*5)	5′-CCAAGTATTGGTTTGTATTTTGCA 3′-CTAGCAAGACCAAAAGGATGTA	Rsa I	151	127, 24
1714C > G	5′-CACTCCTATTGATCTGGTGGA 3′-CCTTATCAAGAGAGAAAGTTTT	Dde I	144, 22	166
1897delC (DPYD*3)	5′-GAACCACCTCTGGCCCCATGTATG 3′-TGTTAAATCACACTTACGTTGTCCG	Msp1	121, 25	146
2194G > A (DPYD*6)	5′-TGGGATGTGAGGGGTGAATG 3′-TTCAGCAACCTCCAAGAAAGC	Mae III	152, 97	249
IVS14 + 1G > A (DPYD*2A)	5′-TGCAAATATGTGAGGAGGGACC 3′-CAGCAAAGCAACTGGCAGATTC	SnaB 1	278, 131	409

substrate was added to the wells and the samples were measured in a photometer at 405 nm (with a reference wavelength of 490 nm). The DPD protein level was determined from a standard curve derived from standard samples in the same ELISA plate.

Statistics

Statistical analysis was performed using the SAS system. One-way ANOVA was used for the group analysis and a *t*-test was used for the gender analysis. *P* values less than 0.05 were considered significant.

Results

Genomic DNA analysis

The frequency of 11 DPD gene mutations in the Taiwanese population was assessed by the PCR-RFLP technique. Among these mutations, four known Asian mutations were found in 300 Taiwanese subjects. These mutations included 4 heterozygous 74A > G mutations, 13 heterozygous 85T > C (DPYD*9A) mutations, 7 heterozygous 2194G > A (DPYD*6) mutations, and 22 homozygous and 126 heterozygous 1627A > G (DPYD*5) mutations. The frequencies of these mutant alleles were 0.67% (4/600), 2.17% (13/600), 1.17% (7/600), and 28.3% (170/600), respectively (Fig. 1, Table 2). There were no combined mutations in these samples except in two subjects with combined heterozygous 85T > C (DPYD*9A) and 1627A > G (DPYD*5) mutations. No mutations were detected in these 300 samples in the other seven mutant alleles, including 62G > A, 812del T, 1003 G > T, 1156G > T, 1714C > G, 1897delC (DPYD*3), and IVS 14 + 1G > A.

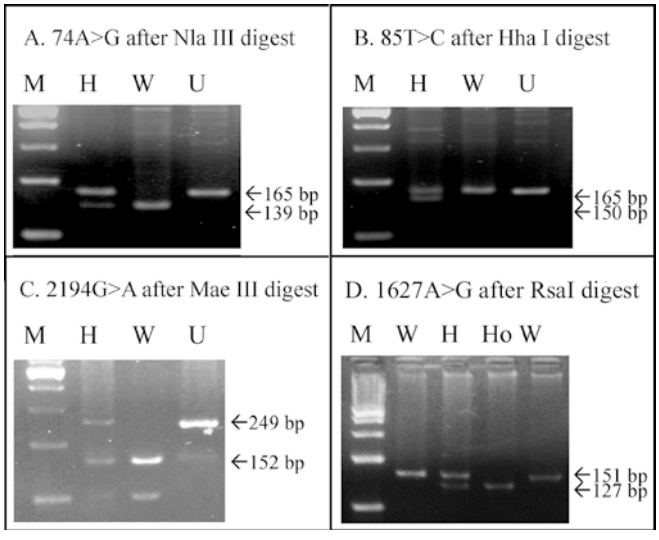

Fig 1 Detection of DPD mutations by the PCR-RFLP method. Four mutations were detected: 74A > G (**A**), 85T > C (DPYD*9A) (**B**), 2194G > A (DPYD*6) (**C**), and 1627 > G (DPYD*5) (**D**). (*M* 100 bp marker, *H* heterozygous mutation, *Ho* homozygous mutation, *W* wildtype, *U* untreated sample)

cDNA sequence analysis

For the mutations found, samples were confirmed using an autosequencer. There were truly mutations over these four mutant sites (data not shown).

DPD level analysis

DPD protein levels were determined in samples from 72 healthy volunteers. These samples included 7 homozy-

Table 2 Mutant frequency of the 11 alleles in Taiwanese population

Mutation	Frequency		Effect of genotype
	%	/600	
62G > A	0	0	Arg to Gln
74A > G	0.67	4	His to Arg
85T > C (DPYD*9A)	2.17	13	Cys to Arg
812delT	0	0	Frameshift
1003G > T	0	0	Val to Leu
1156G > T	0	0	Glu to terminal
1627A > G (DPYD*5)	28.3	170	Ile to Val
1714C > G	0	0	Leu to Val
1897delC (DPYD*3)	0	0	Frameshift > terminates at codon 633
2194G > A (DPYD*6)	1.17	7	Val to Ile
IVS14 + 1G > A(DPYD*2A)	0	0	Exon 14 skipped > truncated protein

Table 3 DPD protein levels

	Mean DPD protein level (U/mg protein)	*P* value
1627 mutant type ($n = 70$)		
Homozygous mutation ($n = 7$)	40.66	0.201
Heterozygous mutation ($n = 28$)	28.57	
Normal sequence ($n = 35$)	30.46	
Gender ($n = 72$)		
Male ($n = 34$)	29.48	0.559
Female ($n = 38$)	31.72	

gous, 30 heterozygous, and 35 normal type at the 1627 allelic position. Among these, two samples had combined mutations with heterozygous 1627A > G (DPYD*5) and 85T > C (DPYD*9A). Mutations 62G > A, 74A > G, 812delT, 1003G > T, 1156G > T, 1714C > G, 1897delC (DPYD*3), 2194G > A (DPYD*6), and IVS14 + 1G > A (DPYD*2A) were not found in these 72 samples. DPD protein levels were highly variable, ranging from 6.44 U/mg protein to 79.11 U/mg protein (mean 30.67 U/mg protein, SD 16.05 U/mg protein). Excluding the two samples with combined mutations, 70 samples were analyzed to determine the relationship between the 1627A > G (DPYD*5) mutation and the DPD level. One-way ANOVA for three different mutant types of the 1627 mutation indicated that there were no differences in DPD protein levels between the homozygous group ($n = 7$, mean 40.66 U/mg protein), the heterozygous group ($n = 28$, mean 28.57 U/mg protein), and the normal sequence group ($n = 35$, mean 30.46 U/mg protein) ($P = 0.201$; Table 3). The DPD protein levels of two combined mutation samples were 17.79 U/mg protein and 17.91 U/mg protein, respectively. The values were about 25% of the normal range. All 72 samples were analyzed using a *t*-test in relation to gender. There was no significant difference in DPD protein levels between males ($n = 34$, mean 29.48 U/mg protein) and females ($n = 38$, mean 31.72 U/mg protein) ($P = 0.559$; Table 3).

Discussion

The DPD gene is a large gene of approximately 150 kb, and consists of 23 exons ranging in size from 69 to 1404 bp, with intron sizes ranging from less than 1 kb to over 20 kb [15, 38, 41]. Because of its strong impact on the catabolism of pyrimidine base and the antitumor agent, 5-FU, many mutations have been reported and surveyed in the hope of predicting patients who might show toxicity to 5-FU therapy. In the study reported here, we investigated 11 previously reported mutations using the PCR-RFLP technique in the Taiwanese population to see if these mutations exist in Taiwanese and the exact mutation frequency. We also determined DPD protein levels to see if there was any correlation between DPD protein levels and different genotypes.

This is the first study in which the mutant frequencies of these 11 mutations have been determined in a large Taiwanese population. Among these 11 mutations, 4 known mutations were found in 300 Taiwanese subjects. These included 4 heterozygous 74A > G mutations (frequency of mutant allele 0.67%, 4/600), 13 heterozygous 85T > C (DPYD*9A) mutations (frequency of mutant allele 2.17%, 13/600), 7 heterozygous 2194G > A (DPYD*6) mutations (frequency of mutant allele 1.17%, 7/600), and 22 homozygous and 126 heterozygous 1627A > G (DPYD*5) mutations (frequency of mutant allele 28.3%, 170/600; Table 2). In these 300 samples the mutations 62G > A, 812del T, 1003G > T, 1156G > T, 1714C > G, 1897delC (DPYD*3), and IVS14 + 1G > A (DPYD*2A) were not detected.

DPD protein levels were determined in different 1627A > G (DPYD*5) genotypes. However, no significant differences were found between genotypes and phenotypes ($P = 0.201$). These results, in accordance with those of previous studies, suggested that the 1627A > G (DPYD*5) mutation was a common polymorphism and was not associated with DPD activity [21, 29]. Furthermore, no significant differences were found between males and females ($P = 0.556$). There were two patients with heterozygous 1627A > G (DPYD*5) and 85T > C (DPYD*9A) mutations. Excluding the 1627A > G (DPYD*5) mutations due to the suspected polymorphism in this mutant allele, the DPD levels in these two patients were only about 25% of the range. These levels were low but should not be considered a profound deficiency (i.e. < 5%) [1]. These results suggest that the heterozygous 85T > C (DPYD*9A) mutation may not cause severe DPD deficiency. However, we determined only the DPD protein level and not the DPD activity itself. The real impact of the 85T > C (DPYD*9A) mutation in 5-FU catabolism was not so clear from our data. The compound mutations found in our study also imply that some of the patients may have had more than one variant in the DPD gene. This presentation may hinder the attempt to determine the degree of correlation in the relationship between genotype and phenotype in the 5-FU pharmacogenetics syndrome.

Further advanced methods for genotype analysis are therefore warranted for these complicated mutations.

The 1627A > G (DPYD*5) mutation found in this study is the point mutation of A to G at the 1627 position (codon 543) with a protein change from Ile to Val. Some early studies showed that the mutation may be linked to 5-FU toxicity, but further investigations and our study suggested it to be a common polymorphism associated with a wide range of DPD activities in mononuclear cells [4, 21, 29]. In this study the frequency of the mutant allele was 28.3%, which is similar to that in Japanese (35.2%), African Americans (22.7%), and Caucasian subjects (28%) [21, 29, 38]. However the mutation is less common in Finnish subjects (7%) [39]. The 85T > C (DPYD*9A) mutation, which results in a missense mutation at codon 29 with a protein change from Cys to Arg, has been shown in an in vivo study to be associated with low DPD activity [21]. Although the mutation was rare in this study, it did exist with an allelic frequency of 2.17%. The frequency is also similar to that in Japanese subjects (3.7%) [38, 39]. Due to the small number with this mutation in our study and the fact that the DPD protein level rather than the DPD activity itself was measured, we gained no clear picture of the influence of this mutation on DPD activity. However, our data imply that the heterozygous 85T > C (DPYD*9A) mutation would not cause profound DPD protein deficiency. This result is similar to those of other studies, which suggest that the 85T > C (DPYD*9A) mutation is a common polymorphism [4].

Other mutations found in our study were 74A > G and 2194G > A (DPYD*6) with a protein change from His to Arg at codon 25 and Val to Ile at codon 732, respectively. The 74A > G mutation was first reported in a Japanese population with a frequency of 0.46%, which is similar to ours (0.67%). However, the real influence of this mutation on DPD activity is unclear. The frequency of 2194G > A (DPYD*6) is 5.8% in Caucasians and 4.4% in Japanese, but lower in African Americans (1.9%), which is closer to our result (1.17%) [38]. However, only the homozygous state of 2194G > A (DPYD*6), and not the heterozygous state, has been associated with low DPD activity [14, 29].

The IVS14 + 1G > A (DPYD*2A), 1897delC (DPYD*3), 1003G > T, and 1156 G > T mutations, which have been shown to cause DPD activity deficiency in in vivo studies [17, 19, 21, 22, 24, 28, 36], were not found in our study. The G to A transition at intron 14 + 1, that causes a faulty splice and makes a 165 bp deletion that produces a truncated protein, is thought to be the most common mutation associated with 5-FU toxicity in Europeans [1, 35]. The frequency of this mutation is as high as 2.2% in the Finnish population and 2.7% in Taiwanese [38]. However, we did not find this mutant allele in any of our 300 subjects. This finding is in accordance with that of Wei et al. [39] who did not find the IVS14 + 1G > A (DPYD*2A) splicing mutation in Taiwanese, Japanese or African Americans in a large population survey. This mutation has also not been found in Caucasian subjects [29]. Although this mutation is considered to be important in 5-FU treatment in other ethnic groups, the rare frequency in the Taiwanese population indicates that it is unlikely to be related to 5-FU toxicity in Taiwan. Deletion of C at position 1897, which causes a frameshift with low DPD activity, was also not detected in our study. This result is similar to that of Ridge et al. [29] in 60 Caucasian subjects. In Japanese patients with severe toxicities, 1003G > T and 1156G > T are novel mutations. Though these mutations are associated with low DPD activity, especially the association of 1156G > T with undetectable activity, these two mutations are rare and were not found in our study. Although these four mutations have an important impact on DPD activity, they are rare in the Taiwanese population.

DPD protein levels were determined using a DPD ELISA kit in blood samples collected from 72 patients at the same time of day to avoid circadian effects [11, 23]. DPD protein levels were highly variable, ranging from 6.44 to 79.11 U/mg protein (12.28-fold) with a mean of 30.66 U/mg protein. Because the standard enzyme assay is complicated and was not available in our center, we used a DPD ELISA kit. However, the large variations in DPD protein levels were similar to those found using the DPD activity assay in other studies [2, 25, 34]. Our data suggest that the 1627A > G (DPYD*5) mutation is a polymorphism, in accordance with other reports [21, 29]. The finding of no significant difference in DPD protein levels between males and females is also in accordance with other reports [2]. However, in some studies a difference in activity between genders has been found, but the difference was so small that it was thought to have no clinical impact on 5-FU metabolism in patients with cancer [29, 30, 32].

Two samples with combined heterozygous 1627A > G (DPYD*5) and 85T > C (DPYD*9A) mutations showed low DPD protein levels (about 25% of the normal range), but not a profound deficiency (i.e. < 5%) [1]. Although early findings indicated that the 85T > C (DPYD*9A) mutation may be associated with low DPD activity, our findings suggest that the heterozygous mutation does not result in profound deficiency of DPD protein levels. However, complicated mutations with more than one mutant allele, as in these two samples, hinder the establishment of a clear correlation between genotype and phenotype. Thus, the use of a single genotyping assay as a method to identify DPD-deficient patients is precluded, and there may be some controversy about the results of genotype and phenotype analyses [1, 18, 35]. A more comprehensive method such as denaturing high-performance liquid chromatography analysis is necessary to identify both known and unknown sequence variations in this complicated pharmacogenetic syndrome [8, 9].

In summary, 11 known Asian DPD gene mutations were investigated in a Taiwanese population. Four mutations, including 74A > G, 85T > C (DPYD*9A), 1627A > G (DPYD*5), and 2194G > A (DPYD*6), were found in 300 subjects. The mutations 62G > A, 812delT,

1003G > T, 1156G > T, 1714C > G, 1897delC (DPYD*3), and IVS14 + 1G > A (DPYD*2A) were not detected. These results reveal some ethnic differences in DPD gene mutations. The phenotype analysis in terms of DPD protein levels suggested that the 1627A > G (DPYD*5) mutation is not associated with DPD protein level and might be a polymorphism in the DPD gene. DPD levels were also not correlated with gender in this study. No significant correlation between these 11 mutations and DPD protein levels was found, indicating that investigation of these mutations is insufficient to predict toxicity in Taiwanese patients scheduled to receive 5-FU. Therefore, further investigation of a new comprehensive method and identification of other molecular alterations are warranted to facilitate clinical practice in 5-FU pharmacogenetic syndrome.

Acknowledgement This work was supported by grant NSC89-2314-B-037-103 from the National Science Council, Taiwan.

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