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## ORIGINAL INVESTIGATION

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## Dihydropyrimidine dehydrogenase (DPD) deficiency: identification and expression of missense mutations C29R, R886H and R235W

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**Abstract** Dihydropyrimidine dehydrogenase (DPD) deficiency (McKusick 274270) is an autosomal recessive disease characterized by thymine-uraciluria in homozygous-deficient patients and associated with a variable clinical phenotype. Cancer patients with this defect should not be treated with the usual dose of 5-fluorouracil because of the expected lethal toxicity. In addition, heterozygosity for mutations in the DPD gene increases the risk of toxicity in cancer patients treated with this drug. Sequence analysis in a patient with complete DPD deficiency, previously shown to be heterozygous for the  $\Delta$ C1897 frameshift mutation, revealed the presence of a novel missense mutation, R235W. Expression of this novel mutation and previously identified missense mutations C29R and R886H in *Escherichia coli* showed that both C29R and R235W lead to a mutant DPD protein without significant residual enzymatic activity. The R886H mutation, however, resulted in about 25% residual enzymatic activity and is unlikely to be responsible for the DPD-deficient phenotype. We show that the *E. coli* expression system is a valuable tool for examining DPD enzymatic variants. In addition, two new patients who were both heterozygous for the C29R mutation and the common splice donor site mutation were identified. Only one of these patients showed convulsive disorders during childhood, whereas the other showed no clinical phenotype, further illustrating the lack of correlation between genotype and phenotype in DPD deficiency.

### Introduction

Dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) catalyzes the first and rate-limiting step in the catabolism

of uracil, thymine and the analog 5-fluorouracil (Lu et al. 1993). Patients do not exhibit a characteristic clinical phenotype, although in about half of the cases with near-complete deficiency of the enzyme convulsive disorders are observed (Berger et al. 1984; Braakhekke et al. 1987; Van Gennip et al. 1987, 1989, 1994, 1997a, b). In patients with a nearly complete enzymatic defect, the initial diagnosis can be made on the presence of large amounts of both thymine and uracil in the patients' body fluids, whereas the diagnosis can be confirmed by measurement of the enzymatic activity in either peripheral mononuclear cells or fibroblasts (Van Gennip et al. 1995; Van Kuilenburg et al. 1997). In addition to this category of patients, there are cancer patients experiencing acute 5-fluorouracil toxicity due to lower than normal DPD activity. In these cases, however, deficiency of DPD is in most cases only partial with residual enzymatic activities ranging from 6% to 54% (Diasio et al. 1988; Harris et al. 1991; Van Kuilenburg et al. 1997). Recently, it was demonstrated that such patients are heterozygous for a genomic DPD mutation and it was advocated that patients receiving 5-fluorouracil should be screened for the presence of this mutation prior to therapy in order to avoid severe toxicity (Wei et al. 1996; Van Kuilenburg et al. 1997).

The human DPD enzyme consist of two identical subunits of 111 kDa and is encoded by a single gene mapped to chromosome 1p22 (Takai et al. 1994; Yokota et al. 1994). The recent cloning of the DPD cDNA and publication of parts of the sequence of the genomic DPD gene now allows detection of the defect at the molecular level (Yokota et al. 1994; Vreken et al. 1996a; Johnson et al. 1997). So far, four mutations in the DPD gene have been described. The first mutation is a G→A point mutation in the invariant GT splice donor sequence in intron 14, leading to skipping of the 165-bp exon immediately upstream of the splice donor site mutation (Meinsma et al. 1995; Vreken et al. 1996a). This mutation has been identified in 11 out of 18 patients with complete DPD-deficiency in either the homozygous or heterozygous state and has apparently a high frequency among DPD-deficient individuals from northern Europe (Vreken et al. 1996a, b). In addi-

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tion, heterozygosity for this mutation was identified in patients experiencing acute 5-fluorouracil toxicity (Wei et al. 1996; Van Kuilenburg et al. 1997). Two other mutations, a frameshift ( $\Delta$ C1897) and a missense (C29R) mutation, were identified in two patients from Turkey (Vreken et al. 1996b, 1997a). Finally, a four-base deletion ( $\Delta$ TCAT<sub>296-299</sub>) was identified in two independent families with DPD deficiency (Vreken et al. 1997b).

In this paper we describe a novel missense mutation (R235W). Using an *Escherichia coli* system to express the R235W and the previously identified C29R and R886H mutations, we clearly show that both the R235W and C29R mutations lead to an inactive DPD protein.

## Materials and methods

### Isolation of RNA and DNA

RNA and DNA were isolated from cultured fibroblasts by the guanidinium thiocyanate method (Chomczynski and Sacchi 1987) and standard procedures, respectively (Hoar 1987).

### Polymerase chain reaction (PCR) analysis

cDNA synthesis and reverse-transcription-PCRs (RT-PCRs) for amplifying DPD cDNA were carried out essentially as described previously (Vreken et al. 1996a, 1997b; Van Kuilenburg et al. 1997). Four overlapping DPD cDNA fragments were amplified using sense primers AF, BF, CF and DF corresponding to nucleotides -21-1, 606-629, 1473-1496 and 2254-2276, respectively and antisense primers AR, BR, CR and DR complementary to nucleotides 694-716, 1590-1616, 2353-2376 and 3145-3169, respectively (ATG = 1). Forward primers contained a 5'-TGT-AAAACGACGGCCAGT-3' extension whereas reverse primers contained a 5'-CAGGAAACAGCTATGACC-3' extension at their 5' ends. The latter sequences are complementary to fluorescent-labeled primers used in the dye terminator sequence reaction. Amplification was carried out in 50- $\mu$ l reaction mixtures containing 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 1  $\mu$ M of each primer, 0.2 mM dNTPs. After initial denaturation for 5 min at 95°C, 2 U of *Taq* polymerase (AmpliTaq, Perkin Elmer, San Jose, Calif.) was added and amplification carried out for 30 cycles (1 min 95°C, 1 min 65°C, 1 min 72°C).

Amplification of the genomic region containing a DPD exon and its flanking sequences was carried out as described previously (Vreken et al. 1996a). PCR products were purified using a Qiquickspin PCR purification kit (Qiagen, Hilden, Germany) and used for direct sequencing.

### Sequence analysis

Sequence analysis was carried out on an Applied Biosystems model 377 automated DNA sequencer using the dye-primer method for the DPD cDNA and genomic fragments (dye-primer cycle-sequence-ready reaction kit, Applied Biosystems, San Jose, Calif.).

### Assay of DPD activity

DPD activity in fibroblasts and crude *E. coli* lysates was measured as described previously (Van Kuilenburg et al. 1996).

### Construction of expression plasmids

Expression plasmids were constructed in the vector pSE420 (Invitrogen, San Diego, Calif.) using the human DPD cDNA subcloned

in a pUC19 vector (a kind gift of Dr. F.J. Gonzalez, NIH). First, part of the polylinker sequence in pSE420 between the *Sal*I and *Hind*III sites was deleted by cleavage with these enzymes and religation. The resulting plasmid, pSE420 $\Delta$ SH, was cut with *Nco*I and *Eco*RI and the DPD cDNA *Nco*I-*Eco*RI fragment, spanning the entire coding region and part of the 3'-untranslated region up to the *Bsp*HI site (the *Eco*RI site is derived from pUC19) was ligated, resulting in the plasmid pSE420 $\Delta$ SH-DPD. In order to introduce the C29R or R235W mutation, the 886-bp *Nco*I-*Eco*NI fragment from the AF-BR cDNA amplification product derived from either patient A or patient B was ligated into the *Nco*I-*Eco*NI-digested pSE420 $\Delta$ SH-DPD plasmid. The resulting clones were designated pSE420 $\Delta$ SH-DPD-C29R and pSE420 $\Delta$ SH-DPD-R235W, respectively.

The 277-bp *Bam*HI-*Pst*I fragment from the CF-DR cDNA amplification product derived from patient A was ligated into *Bam*HI-*Pst*I-digested pSE420 $\Delta$ SH-DPD plasmid, resulting in pSE420 $\Delta$ SH-DPD-R886H. The same fragment was ligated into pSE420 $\Delta$ SH-DPD-C29R, resulting in the plasmid pSE420 $\Delta$ SH-DPD-C29R/R886H. All clones were sequenced completely in order to verify the presence of the mutation and exclude the presence of random mutations introduced by PCR artifacts. One clone harbored the mutation G122A, leading to the nonsense alteration W41X. This construct was used as a negative control in the expression experiments and designated pSE420 $\Delta$ SH-DPD-W41X.

### Expression of missense mutations in *E. coli*

Expression plasmids were introduced into *E. coli* strain BL21(F-, *ompT*, *hdsS<sub>b</sub>(r<sub>b</sub>m<sub>b</sub>)*, *gal*, *dcm*). A 20-ml LB broth culture, supplemented with 100  $\mu$ g/ml ampicillin, 100  $\mu$ M uracil, 100  $\mu$ M each of FAD and FMN, and 10  $\mu$ M each of Na<sub>2</sub>S and Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>) was inoculated with 200  $\mu$ l of an overnight preculture grown in LB broth. Cells were grown to OD<sub>550</sub> = 0.5-0.6 at 30°C and induction was performed by the addition of 1 mM isopropyl-1-thio-galactopyranoside. Cells were sedimented at time points between 1 and 3 h, washed with isolation buffer (35 mM potassium phosphate, pH 7.3, 10 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ M leupeptin and 2  $\mu$ M pepstatin) and resuspended in isolation buffer. The cell suspension was frozen at -20°C for at least 16 h, thawed on ice and lysed by sonication. The crude lysate was centrifuged at 20,000 g for 15 min. The resulting supernatant was used for the DPD activity assay using the same method as previously applied to fibroblast homogenates (Van Kuilenburg et al. 1996), Laemmli gel electrophoresis or protein quantitation (bicinchoninic acid procedure, Pierce, Rockford, Ill.).

### Analysis of expressed DPD protein

Analysis of DPD protein was carried out by Laemmli gel electrophoresis followed by Western immunoblotting essentially as described previously (Yokota et al. 1994). The Western blot was incubated with a polyclonal anti-pig DPD antibody (a kind gift of Dr. F. J. Gonzalez) followed by [<sup>125</sup>I]protein A incubation using standard procedures (Maniatis et al. 1982). Immunoreactive DPD was visualized on a Molecular Dynamics  $\beta$ -imaging system.

## Results

### Clinical and biochemical characterization

Detailed clinical descriptions of patients A and B and their family members have been published elsewhere (Vreken et al. 1997a). These patients were shown to be completely DPD deficient as judged from DPD enzymatic studies in cultivated fibroblasts, and the father of patient

**Table 1** Genotype and dihydropyrimidine dehydrogenase (DPD) enzymatic activity. (*nd* not detectable, *wt* wild type, *fr* frameshift, *del* deletion)

Patient	DPD activity <sup>a</sup>	Genotype	Effect <sup>b</sup>
A	0.0015	$\Delta$ C1897/C703T	fr/R235W
Father A	nd	$\Delta$ C1897/ $\Delta$ C1897	fr/fr
Mother A	0.47	C703T/wt	R235W/none
B	nd	T85C,G2658A/T85C, G2658A	C29R,R886H
C1	nd	T85C/IVS14 (G+1→A)	C29R/del(exon 14)
C2	nd	T85C/IVS14 (G+1→A)	C29R/del(exon 14)

<sup>a</sup>DPD enzymatic activity measured in fibroblasts is expressed as nanomoles product per hour per milligram protein. (control values  $1.12 \pm 0.7$ , mean  $\pm$  1 SD,  $n = 15$ )

<sup>b</sup>Effect of the mutation on DPD protein or mRNA

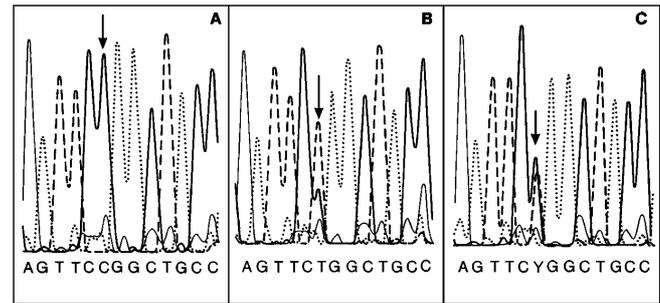
A was also completely DPD deficient. The mother of patient A showed intermediate DPD activity in accordance with her obligate carrier status (Table 1). Patient C1 showed epileptic manifestations during childhood but is now, at the age of 30 years, clinically normal. Her sister (patient C2), aged 27 years, is also completely DPD deficient, but has never shown any clinical signs. The parents of patients B and C were not available for study.

#### Genomic sequence analysis

Since we had previously identified a splice donor site mutation in two unrelated Dutch patients, we tested the patients and the parents of patient A for this mutation. A 333-bp genomic DNA fragment encompassing a DPD exon and its flanking intron sequences was amplified and directly sequenced. The previously reported splice donor site mutation (Vreken et al. 1996a) was detected in patient C1 and her sister (patient C2) in the heterozygous state (data not shown). Patient A was shown to be heterozygous for a single base pair deletion at position 1897, whereas his father was homozygous for the  $\Delta$ C1897 mutation. This mutation leads to a frameshift and a premature stop codon shortly thereafter (Vreken et al. 1997a). The mother of patient A showed no abnormalities in this genomic region (data not shown).

#### cDNA sequence analysis

Total RNA isolated from fibroblasts of the patients and the parents of patient A was subjected to RT-PCR. The mother of patient A was shown to be heterozygous for a novel single-base substitution, C703T, leading to the amino acid substitution R235W (Fig. 1). Patient A was previously shown to be heterozygous for the single-base deletion  $\Delta$ C1897 (Vreken et al. 1997a). This allele exhibits reduced stability, as judged from the very weak signal obtained from the father of this patient who is homozygous for this mutation (Vreken et al. 1997a). In ac-



**Fig. 1** Identification of the C703T (R235W) mutation. Direct sequence analysis was performed on amplified cDNA fragments in a control (A), patient A (B) and the mother of patient A (C). The C703T mutation is indicated with an arrow. Note that the C/T ratio at position 703 is 1:3 in B and 1:1 in C. For explanation see text

cordance with this observation, sequence analysis in patient A showed an approximately 3:1 ratio for T:C at position 703, implying that the steady-state concentration of the R235W allele in this patient is significantly higher than that of the  $\Delta$ C1897 allele (Fig. 1).

Patient B is homozygous for both the T85C and G2658A mutations, leading to amino acid substitutions C29R and R886H (Vreken et al. 1997a). Patient C1 and her sister (C2) were shown to be heterozygous for the T85C mutation and are therefore compound heterozygotes for the G→A splice site mutation, leading to skipping of exon 14, and the C29R mutation (cf. Table 1).

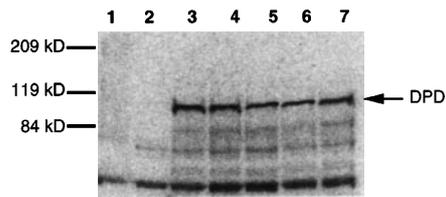
#### Expression of missense mutations

In order to establish whether the missense mutations C29R, R886H and R235W are responsible for the DPD-deficient phenotype, these mutations were introduced in the pSE420 $\Delta$ SH-DPD expression vector (see Materials and methods). The endogenous DPD enzymatic activity in the *E. coli* strain used for expression of the constructs was below the limit of detection of the assay (0.01 nmol/mg protein per hour; Van Kuilenburg et al. 1996). Introduction of the wild-type DPD construct increased DPD activity more than 1,000-fold above background (Table 2). Expression of

**Table 2** Enzymatic activity of expressed mutant DPD

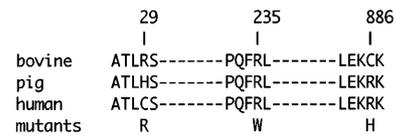
Construct	DPD enzymatic activity <sup>a</sup> (mean $\pm$ 1 SD, $n = 4$ )
pSE420 $\Delta$ SH	< 0.01
pSE420 $\Delta$ SH-DPD	16.7 $\pm$ 1.5
pSE420 $\Delta$ SH-DPD-W41X	< 0.01
pSE420 $\Delta$ SH-DPD-C29R	< 0.01
pSE420 $\Delta$ SH-DPD-R886H	4.4 $\pm$ 0.4
pSE420 $\Delta$ SH-DPD-C29R/R886H	< 0.01
pSE420 $\Delta$ SH-DPD-R235W	0.2 $\pm$ 0.05

<sup>a</sup>DPD enzymatic activity measured in *Escherichia coli* lysates is expressed as nanomoles product per hour per milligram protein



**Fig. 2** Western immunoblot analysis of expressed mutant DPD proteins. Sonicated crude *E. coli* lysates from cells expressing (mutant) DPD cDNAs were subjected to electrophoresis and transferred to a nitrocellulose membrane. DPD was stained using a pig polyclonal anti-DPD antibody and [<sup>125</sup>I]protein A. Lane 1 pSE420ΔSH, 2 pSE420ΔSH-DPD-W41X, 3 pSE420ΔSH-DPD, 4 pSE420ΔSH-DPD-R235W, 5 pSE420ΔSH-DPD-C29R/R886H, 6 pSE420ΔSH-DPD-R886H, 7 pSE420ΔSH-DPD-C29R. Size markers are indicated on the left side of the blot. The signals in the lower part of the blot (35 and 60 kDa) result from cross-reactivity with *E. coli* proteins (Yokota et al. 1994)

pSE420ΔSH-DPD-C29R, pSE420ΔSH-DPD-C29R/R886H and the construct pSE420ΔSH-DPD-W41X yielded no detectable DPD activity, whereas pSE420ΔSH-DPD-R886H and pSE420ΔSH-DPD-R235W showed about 25% and 1% residual activity, respectively (Table 2). In order to check that all mutant proteins were expressed properly, we subjected the *E. coli* lysates to Laemmli gel electrophoresis. Coomassie blue staining of the protein gel showed no substantial amounts of DPD protein in either wild-type or mutant crude lysates when compared with the vector alone or the W41X construct, indicating that only a low amount of DPD protein is present in the *E. coli* lysates (data not shown). Detection of DPD protein required Western blotting and hybridization with a polyclonal antibody against pig DPD. This polyclonal antibody cross-reacts with several *E. coli* proteins of between 35 and 65 kDa in size (Yokota et al. 1994). Although this type of analysis does not allow a quantitative determination of the amount of (mutant) DPD protein, Fig. 2 shows that all full-length mutant DPD proteins are synthesized in the *E. coli* host at roughly the same concentration as the wild-type construct. In addition an 80-kDa DPD degradation product is present in lanes 3–7. Neither the vector alone nor the truncated DPD mutant W41X shows a signal at the position where mature DPD protein or the 80-kDa degradation product is expected on the immunoblot, whereas the 35-kDa and 60-kDa nonspecific bands are detectable in the control lysates although at lower intensities than in lanes 3–7 (Fig. 2). Longer exposures of the immunoblot showed no DPD-specific bands in either lane 1 or 2 (data not shown). The observed expression of mutant DPD protein implies that the lack of enzymatic activity in the constructs pSE420ΔSH-DPD-C29R, pSE420ΔSH-DPD-C29R/R886H and pSE420ΔSH-DPD-R235W is not caused by rapid degradation of the mutant proteins in the *E. coli* lysate. Therefore we conclude that C29R and R235W are the causative mutations. Based on the measurements of DPD activity expressed per milligram protein, the reduction of DPD activity in the R886H construct is about 75%. However, since the immunoblot shows slight variation in the level of expression between the



**Fig. 3** Partial sequence comparison of bovine, pig and human DPD peptide sequences. Mutant positions 29, 235 and 886 are indicated. Alignment was performed using the BCM search launcher (Smith et al. 1996)

wild-type and mutant constructs it is justifiable to conclude only that R886H alone is not sufficient to explain the DPD-deficient phenotype in patient B but reduces DPD catalytic activity.

## Discussion

Mutation analysis in four DPD-deficient patients revealed the presence of one frameshift mutation (ΔC1897) and three amino acid substitutions (C29R, R235W and R886H). The frameshift mutation leads to premature termination of translation before the uracil-binding site, which is encoded by nucleotides 1960–1993 (Yokota et al. 1994). Therefore it is clear that the mutant protein cannot express any residual DPD activity in accordance with the absence of activity as observed in fibroblasts of the father of patient A (Table 1). Analysis of the missense mutations in an *E. coli* expression system revealed that both C29R and R235W result in an inactive mutant DPD protein. Moreover, the mutant DPD proteins are stable in the *E. coli* lysate as judged by immunoblot analysis. The R886H mutation does decrease the catalytic activity of the resulting mutant DPD protein but is unlikely to be responsible for the lack of residual activity in patient B, who is homozygous for both the C29R and R886H mutations. So far, screening of 18 unrelated DPD-deficient individuals did not reveal the latter mutation in any patient. Screening for this mutation in the general population and in a group of patients experiencing acute 5-fluorouracil toxicity is currently in progress.

Alignment of the DPD sequences from pig, bovine and human revealed that the arginine residue at position 235 is conserved between the three species, but that the cysteine at position 29 and the arginine at position 886 are not (Fig. 3). The spacing of cysteine residues is, however, different in human, bovine and pig DPD (Yokota et al. 1994). Therefore, an altered secondary or tertiary structure caused by different disulfide bridging that abolishes enzymatic activity is the most likely explanation for the fact that the C29R mutation causes a lack of residual enzymatic activity. Apparently the R886H mutation, located outside the catalytic domains of the DPD protein, also causes a conformational change that leads to reduced enzymatic activity. The *E. coli* expression system presented here is a valuable tool for examining enzymatic DPD variants obtained through screening of DPD-deficient individuals, clearly discriminating between mutations leading to a complete or partially deficient DPD protein.

Previous studies have revealed that the correlation between phenotype and genotype in DPD deficiency is not very strong (Fernandez-Salguero et al. 1997; Vreken et al. 1997a, b). Homozygosity for a four-base deletion (delTCAT296-299) in the DPD gene was observed in a patient suffering from microcephaly and convulsive disorders, whereas the same genotype did not lead to a clinical phenotype in the brother of this patient (Vreken et al. 1997b). In a family of Pakistani origin, a patient, homozygous for the common splice donor site mutation, showed developmental delay and motor impairment, whereas his brother showed the same genotype but no clinical symptoms (Fernandez-Salguero et al. 1997). In this study also, there seems to be no correlation between the clinical phenotype and genotype in DPD-deficient patients. Patient C2, who is completely DPD deficient, has never showed any clinical signs, whereas her sister (patient C1) showed epileptic manifestations during childhood. In addition, the father of patient A proved to be homozygous for the  $\Delta$ C1897 frameshift mutation but had no known history of convulsive disorders. The lack of correlation between genotype and phenotype in these families cannot easily be explained. However, both population screening based on DPD catalytic activity and a limited study screening for the common splice donor site mutation revealed that as much as 3–5% of the normal population could be heterozygous for mutant DPD alleles (Lu et al. 1993, 1995; McMurrough and McLeod 1996; Wei et al. 1996). Because these studies suggest a 10- to 20-fold higher frequency (up to 1:1,000) for either homozygosity or compound heterozygosity for DPD mutations than is observed in regular metabolic screening of children with convulsive disorders, it is tempting to speculate that the vast majority of DPD-deficient individuals show no clinical phenotype. The study presented here provides more evidence that indeed some DPD-deficient patients at least show no characteristic clinical phenotype. Apparently, additional factors must play an important role in expression of the clinical phenotype. It is possible that a second gene, closely linked to the DPD gene on chromosome 1, is required for full clinical manifestation of the disorder, but so far such a gene has not been identified (Fernandez-Salguero et al. 1997). Another factor might include  $\beta$ -alanine homeostasis, since this neurotransmitter cannot be synthesized and can only be derived from dietary sources in DPD-deficient individuals (Van Gennip et al. 1997a; Van Kuilenburg et al. 1996). The relation between  $\beta$ -alanine homeostasis and clinical symptoms is currently under investigation in our laboratory.

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