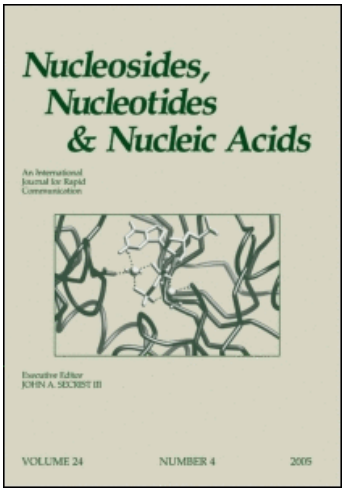

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article van Kuilenburg, A. B. P. , Meijer, J. , Dobritzsch, D. , Lohkamp, B. , Ruitenbeek, W. , Roelofsen, J. , Abeling, N. G. G. M. , Duran, M. and Buzing, C.(2008) 'Identification of Two Novel Mutations C79X and R235Q in the Dihydropyrimidine Dehydrogenase Gene in a Patient Presenting With Hematuria', Nucleosides, Nucleotides and Nucleic Acids, 27: 6, 809 — 815
To link to this Article: DOI: 10.1080/15257770802146247
URL: http://dx.doi.org/10.1080/15257770802146247

Nucleosides, Nucleotides, and Nucleic Acids, 27:809–815, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802146247

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IDENTIFICATION OF TWO NOVEL MUTATIONS C79X AND R235Q IN THE DIHYDROPYRIMIDINE DEHYDROGENASE GENE IN A PATIENT PRESENTING WITH HEMATURIA

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□ *A patient with hematuria was shown to have thymine-uraciluria. The dihydropyrimidine dehydrogenase (DPD) activity in peripheral blood mononuclear cells was 0.16 nmol/mg/h; controls: 9.9 ± 2.8 nmol/mg/h. Analysis of DPYD showed that the patient was compound heterozygous for the novel mutations 237C>A (C79X) in exon 4 and 704G>A (R235Q) in exon 7. The nonsense mutation (C79X) leads to premature termination of translation and thus to a non-functional protein. Analysis of the crystal structure of pig DPD suggested that the R235Q mutation might interfere with the binding of FAD and the electron flow between the NADPH and the pyrimidine substrate site of DPD.*

Keywords Dihydropyrimidine dehydrogenase; *DPYD* mutation; 5-fluorouracil; pharmacogenetics; pyrimidines; uracil; thymine

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases. It catalyzes the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively. DPD deficiency is an autosomal recessive

This study was supported by the "Stichting Sacha Swarttouw-Hijmans," the Wenner-Grenska Samfundet and the Swedish Cancer Foundation.

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disease characterized by thymine-uraciluria in patients with a complete enzyme deficiency. In children, a deficiency of DPD is often accompanied by a neurological disorder but a considerable phenotypic variability has been reported among these patients.[1–4]

DPD is also responsible for the breakdown of the widely used anti-neoplastic agent 5-fluorouracil (5FU). In this light, a partial or complete DPD deficiency is a pharmacogenetic disorder affecting cancer patients who develop severe toxicity, including death, following the administration of 5FU.[5,6] A number of these patients proved to be heterozygous for a mutant allele of *DPYD*.[7–10] Considering the pivotal role of DPD in chemotherapy using 5FU, the identification of novel mutations underlying a DPD deficiency is of utmost importance since it will allow the identification of patients at risk.

The gene encoding DPD (*DPYD*) is present as a single copy gene on chromosome 1p22 and consists of 23 exons.[11] The analysis of *DPYD* in pediatric patients with a complete DPD deficiency has contributed significantly to the identification of disease-causing mutations.[1–3] The resolution of the crystal structure of pig DPD has enabled the prediction of the effects of mutations on the protein conformation and/or the binding of the various cofactors and substrates.[3,12,13] In this paper, we report the identification of two novel mutations in *DPYD* and the analysis of the missense mutation in a three dimensional framework.

MATERIALS AND METHODS

Analysis of Pyrimidine Bases and DPD Activity

The pyrimidine bases uracil and thymine in urine and plasma were analysed using HPLC electrospray tandem mass spectrometry, as described before.[14] The activity of DPD was determined in peripheral blood mononuclear (PBM) cells using radiolabeled thymine followed by separation of radiolabeled thymine from radiolabeled dihydrothymine using reversed-phase HPLC.[15]

Mutation Analysis of DPYD

DNA was isolated from leukocytes using the Wizard Genomic DNA Purification Kit (Promega Benelux, b.v., Leiden, The Netherlands). PCR amplification of all 23 coding exons and flanking intronic regions was carried out using intronic primer sets, as described before.[9] Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems model 3100 automated DNA sequencer using the dye-terminator method (Perkin Elmer Corp., Foster City, CA, USA).

Stereo Views of the Point Mutation Sites

The figures showing the DPD dimer, the point mutation site and its environment were generated using the program PyMOL (W. L. DeLano, The PyMOL Molecular Graphics System (2002) online at http://www.pymol.org).

RESULTS

The patient was a 7-year-old boy who presented with hematuria probably associated with renal calculi. Analysis of a urine sample of the patient for abnormalities in purine and pyrimidine metabolism, primarily aimed at detecting xanthinuria and orotic aciduria, showed strongly elevated levels of uracil (179 μmol/mmol creatinine; controls 7 $\pm$ 6 μmol/mmol creatinine, n = 112) and thymine (103 μmol/mmol creatinine; controls 0.1 $\pm$ 0.3 μmol/mmol creatinine, n = 112). In addition, 5-hydroxymethyluracil was present in the urine of the patient (10 μmol/mmol creatinine), which is normally not detected in urine from healthy volunteers. Highly elevated concentrations of uracil (18 μM; controls <0.4 μM; n = 40) and thymine (13 μM; controls <0.1 μM; n = 40) were detected in plasma as well. The DPD activity in PBM cells was 0.16 nmol/mg/h which was 4 times the value of the baseline noise (controls: 9.9 $\pm$ 2.8 nmol/mg/h; Table 1).

Analysis of *DPYD* for the presence of mutations showed that the patient was heterozygous for a novel 237C>A (C79X) nonsense mutation in exon 4. In addition, the patient was heterozygous for a novel mutation 704G>A (R235Q) in exon 7 (Table 1). Analysis of *DPYD* from the parents showed that the father was heterozygous for the 237C>A (C79X) mutation and that the mother was heterozygous for the 704G>A (R235Q) mutation. The DPD activity in PBM cells of the parents was decreased compared to that observed in controls and comparable to that observed for other obligate heterozygotes (Table 1).

TABLE 1 Mutations and DPD activity

	DPD activity (nmol/mg/h)	Genotype[a]	Effect
Patient	0.16	c. [237C>A] +[704G>A]	p.C79X + p.R235Q
Mother	4.6	c. [704G>A] +[=]	p.R235Q
Father	4.8	c. [237C>A] +[=]	p.C79X
Controls (n = 54)[b]			
Mean $\pm$ SD	9.9 $\pm$ 2.8		
Heterozygotes (n = 20)[b]			
Mean $\pm$ SD	4.8 $\pm$ 1.7		

[a]Mutation nomenclature according to den Dunnen and Antonarakis.[20]
[b]Data taken from van Kuilenburg et al.[21]

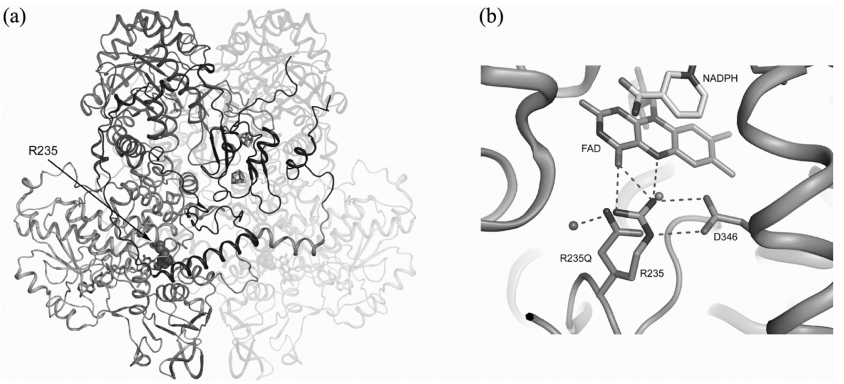

FIGURE 1 The mutation site in the crystal structure of wildtype pig liver DPD. a) Schematic view of the pig liver DPD homodimer with all cofactors shown as stick models. The location of the mutation site is indicated by the space fill representation of the R235 side chain. b) Close-up view of the mutation site, with stick models of the cofactor FAD and the electron donor NADPH. Hydrogen bonding interactions observed in the crystal structure are indicated by dotted lines.

The subunit of mammalian DPD homodimer consists of five domains, and each of them binds at least one of several redox cofactors (FAD, FMN, NADPH, [4Fe-4S]-clusters) employed in the catalysis of the reduction reaction (Figure 1a). R235 is located in the FAD-binding domain and, like most residues in the FAD-binding site, strictly conserved in the mammalian enzymes. The crystal structure available for pig liver DPD reveals that R235 is directly involved in the binding of the cofactor's isoalloxazine ring by formation of hydrogen bonds between the guanidinium group and atoms N5 and O4 of FAD (Figre 1b).[12] A salt bridge to the carboxyl group of D346 fixes the position of the arginine side chain and compensates for its charge. A glutamine side chain is not charged and also shorter than that of arginine. Therefore, the observed mutation R235Q is expected to weaken the binding of the FAD ring system but also to change the electronic environment and hence, the redox potential of the cofactor. Modelling the exchange of R235 to glutamine in the crystal structure of pig liver DPD shows that, assuming the conformation shown in Figure 1b, the carboxamide group of the introduced glutamine would still be within hydrogen bonding distance to atom O4 of FAD. It would, however, be unable to engage in interactions with N5, the acceptor site for the hydride transferred from NADPH, and exclude concomitant interactions with D346. On the other hand, the opposite orientation of the carboxamide group of R235Q is unlikely since it prevents potential hydrogen bonding to FAD. It is possible that the binding of a water molecule at the location modelled in Figure 1b in part compensates for the loss of direct hydrogen bonds by allowing water-mediated interactions. This would explain the residual enzymatic activity of the mutant. Nevertheless, instead of a local positive charge in close proximity to the FAD-N5 there is now nearby the uncompensated negative charge of D346. The resulting

change in redox potential of the flavin cofactor should hamper the electron transfer from the donor NADPH and cause the significantly lower activity of the mutant compared to the wildtype enzyme.

DISCUSSION

DPD deficiency is an autosomal recessive disease biochemically characterised by thymine-uraciluria. In some cases, 5-hydroxymethyluracil, a metabolite of thymine, is also present in urine and plasma.[16] A considerable heterogeneity in the clinical presentation among patients with a DPD deficiency has been observed.[1–4] Motor retardation followed by mental retardation and convulsive disorders were the most abundant manifestations whereas growth retardation, microcephaly, dysmorphy, autism, and ocular abnormalities were less frequently observed.[2,3] In addition, a few patients have been described who, at the time of diagnosis, did not present with any clinical abnormalities.[1,2,4] Thus, the fact that our patient presented only with hematuria is in line with the phenotypic variability of DPD deficiency. Several purine and pyrimidine bases, including xanthine, 2,8-dihydroxyadenine and orotic acid are not very soluble and are associated with renal stones. Since uracil and thymine are very soluble, the combination of a DPD deficiency and hematuria is most likely a coincidence.

A physical map indicated that *DPYD* is at least 950 kb in length with 3 kb of coding sequence and an average intron size of about 43 kb.[11] Numerous mutations have been found in *DPYD* from patients suffering from a (partial) DPD deficiency.[2,3,6] Analysis of *DPYD* showed that the patient was heterozygous for a novel 237C>A nonsense mutation in exon 4, creating a translational stop codon (C79X). This nonsense mutation leads to premature termination of translation before the FAD and uracil/thymine binding sites and thus to a non-functional protein without any residual activity. Furthermore, the patient was heterozygous for a novel mutation 704G>A (R235Q) in exon 7.

DPD appears to be conserved throughout evolution and the high sequence identities (>92%) between human DPD and that of other mammals, such as bovine and pig, suggest that very similar reaction mechanisms and three-dimensional structures exist in these species.[12,17] The elucidation of the crystal structure of pig DPD has opened the possibility to study the effect of missense mutations in a three dimensional framework.[3,12,13] Analysis of the crystal structure of pig DPD suggested that the R235Q mutation might interfere with the binding of FAD and the electron flow between the NADPH and the pyrimidine substrate site of DPD.

Defects in the degradation of pyrimidines have been associated with a variable clinical phenotype whereas the same defects can lead to severe life-threatening toxicities when (partially) deficient individuals are treated with

the pyrimidine analogue 5-fluorouracil.[6,18,19] Thus, carriers for the novel mutations reported in our manuscript possess an increased risk of developing severe toxicity in case they are treated with 5FU.

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