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ORIGINAL INVESTIGATION

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Identification of a four-base deletion (delTCAT_{296–299}) in the dihydropyrimidine dehydrogenase gene with variable clinical expression

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Abstract Dihydropyrimidine dehydrogenase catalyzes the first and rate-limiting step in the breakdown of thymine, uracil, and the widely used antineoplastic drug, 5-fluorouracil. Sequence analysis of the dihydropyrimidine dehydrogenase cDNA in a Dutch consanguineous family identified a novel four-base deletion (delTCAT_{296–299}) leading to premature termination of translation. The deletion is located in a TCAT tandem-repeat sequence and most likely results from unequal crossing-over or slipped mispairing. In this family we identified three homozygous individuals for this mutation. Two of these showed convulsive disorders but one was clinically normal. This observation suggests that, at least in this family, there is no clear correlation between the dihydropyrimidine dehydrogenase genotype and phenotype.

Introduction

Dihydropyrimidine dehydrogenase (DPD) deficiency (McKusick 274270) is a clinically heterogeneous, autosomal recessive disease (Berger et al. 1984; Braakhekke et al. 1987; Van Gennip et al. 1987, 1989, 1994). DPD (EC 1.3.1.2) catalyzes the first and rate-limiting step in the catabolism of uracil, thymine, and the thymine analog, 5-fluorouracil (Lu et al. 1993). Patients with a nearly complete enzyme defect show convulsive disorders in about 50% of cases, whereas patients experiencing acute 5-fluorouracil toxicity usually show DPD enzymatic activities in the heterozygous range. Recently, it was demonstrated that such patients are indeed heterozygous for a genomic DPD mu-

tation (Wei et al. 1996; A. B. P. Kuilenburg et al. unpublished results).

The human DPD enzyme consists 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 a small part of the genomic DPD gene now allows detection of the defect at the molecular level (Yokota et al. 1994; Vreken et al. 1996a). So far, three mutations in the DPD gene have been described. The first mutation is a G→A point mutation in an invariant GT splice donor sequence 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 7 out of 12 patients with complete DPD deficiency in either the homozygous or heterozygous state and has an apparently high frequency among DPD-deficient individuals from northern Europe (Vreken et al. 1996a, b). In addition, this mutation was identified in patients experiencing acute 5-fluorouracil toxicity (Wei et al. 1996; A. B. P. Van Kuilenburg et al., unpublished results). Two other mutations, a frameshift (ΔC1897) and a missense (C29R) mutation, were identified in two patients from Turkey (Vreken et al. 1996b, 1997).

We now report a novel four-base deletion (delTCAT_{296–299}) in a family with DPD deficiency.

Materials and methods

Patients

The family investigated in this study has been extensively described elsewhere (Braakhekke et al. 1987). Briefly, the proband (patient A) was diagnosed having thymine-uraciluria at the age of 4 years. Clinically she showed epileptic manifestations and microcephaly. She is the second child of consanguineous Dutch parents. In addition, her mother is the child of a consanguineous relationship, her father is also her grandfather. From the age of 19 years, the mother suffered from generalized tonic seizures. The brother and father of the proband are clinically normal. In the previous study, the proband, her mother, and brother were shown to be homozygous DPD deficient as judged by enzymatic measurements of the DPD activity in fibroblasts of these patients. The father of

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the proband was shown to have a DPD enzymatic activity in the heterozygous range (Braakhekke et al. 1987).

PCR analysis of DPD cDNA fragments

cDNA synthesis and RT-PCR reactions for amplifying DPD cDNA were carried out essentially as described previously (Meinsma et al. 1995; Vreken et al. 1996a, 1997). Four overlapping DPD cDNA fragments were amplified using sense primers AF, BF, CF, and DF corresponding to nucleotides -21 to -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'-TGTAACGACG-GCCAGT-3' extension whereas reverse primers contained a 5'-CAGGAAACAGCTATGACC-3' extension at their 5' ends. The latter sequences are complementary to fluorescently labeled primers used in the dye terminator sequence reaction (Dye primer cycle sequence ready-reaction kit; Applied Biosystems, San Jose, Calif.). Amplification was carried out in 50- μ l reaction mixtures containing 10 mM TRIS-HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂, 1 μ M of each primer, 0.2 mM dNTPs. After initial denaturation for 5 min at 95°C, 2 U of *Taq* polymerase was added and amplification carried out for 30 cycles (1 min 95°C, 1 min 65°C, 1 min 72°C). Sequence analysis was carried out on an Applied Biosystems 377 automated DNA sequencer.

Restriction analysis

The four-base deletion (delTCAT₂₉₆₋₂₉₉) does not modify a restriction site in the DPD cDNA. Therefore, base substitutions were introduced in the region encompassing the deletion. A sense primer corresponding to nucleotides 144-166 and an antisense primer complementary to nucleotides 299-330, changing A301 to G, T302 to A, and C303 to A were used to amplify a 186-bp cDNA or genomic fragment. The modifications in the antisense primer created a *Bst*BI restriction site in the wild-type allele, but not in the delTCAT₂₉₆₋₂₉₉ allele. Cleavage of amplified fragments with *Bst*BI and subsequent separation in 10% acrylamide gels yields 31- and 155-bp fragments for the wild-type allele, whereas the delTCAT₂₉₆₋₂₉₉ allele yields 182-bp band (Fig. 2).

Results

Total RNA isolated from fibroblasts of the proband and her relatives was subjected to RT-PCR in order to amplify four overlapping regions encompassing the complete DPD cDNA. No deviations in size compared to control samples could be detected (data not shown). Sequence analysis of the amplified fragments revealed that the proband, her mother, and her brother were homozygous for a four-base deletion (delTCAT₂₉₆₋₂₉₉). The father was shown to be heterozygous for this deletion (Fig. 1).

A rapid screening method for this mutation was developed using *Bst*BI restriction analysis of an amplified cDNA fragment encompassing the mutation site (see Materials and methods). The *Bst*BI restriction analysis allows detection of homozygotes and heterozygotes for this mutation at the cDNA level in the family described here (Fig. 2). In addition, this approach yielded similar results when applied to genomic DNA. Therefore, we conclude that the deletion is located within a single exon (data not shown). Screening five complete DPD-deficient patients with uncharacterized mutations revealed one heterozygous individual for the

Fig. 1 A–C Identification of the delTCAT₂₉₆₋₂₉₉ mutation in amplified dihydropyrimidine dehydrogenase (DPD) cDNA. **A** Control. **B** Patient A. **C** Father of patient A. In patient A, homozygosity for the del(TCAT₂₉₆₋₂₉₉) is shown. The father is heterozygous for this mutation. Note that the sequence diverges after the first TCAT sequence in **C** due to heterozygosity for the deletion. The TCAT sequence is indicated with brackets

Fig. 2 *Bst*BI restriction analysis of the delTCAT₂₉₆₋₂₉₉ mutation in amplified cDNA DPD sequences. A 186-bp (wild type) or 182-bp (mutant) fragment was amplified creating a *Bst*BI restriction site in the wild-type but not in the mutant allele. Cleavage with *Bst*BI yields fragments of 31 and 155 bp for the wild-type allele, whereas the mutant allele yields a 182-bp fragment. Lane M size marker, lane 1 control undigested, lane 2 digested control, lane 3 patient B, lane 4 father of patient A, lane 5 mother of patient A, lane 6 patient A. Note that patient B and the father of patient A are heterozygous and patient A and her mother are homozygous for the delTCAT₂₉₆₋₂₉₉ mutation

same mutation (patient B, Fig. 2). Sequence analysis confirmed heterozygosity for the delTCAT₂₉₆₋₂₉₉ mutation in this patient. In addition, the latter patient was also shown to be heterozygous for the common G→A splice site mutation leading to exon skipping (Vreken et al. 1996a).

Discussion

The four-base deletion identified in this study causes a frameshift leading to a premature stop codon shortly thereafter. Since the premature stop codon is positioned before the NADPH and uracil binding site, encoded by nucleotides 1001-1053 and 1960-1993, respectively (Yokota et al. 1994), it is clear that the truncated mutant

DPD protein cannot express any residual activity, in accordance with the undetectable levels of DPD activity in fibroblasts from the homozygous individuals studied here (Braakhekke et al. 1987).

The δ TCAT₂₉₆₋₂₉₉ mutation is part of a TCAT tandem repeat. Repeat sequences are often involved in DNA rearrangements (Chandley 1989). Either slipped mispairing or unequal chromosome crossing-over were suggested to be the likely mechanisms for the generation of such mutations (Efstratiadis et al. 1980; Smith 1976).

Patient A, her mother, and her brother show the same DPD genotype and all three lack any DPD enzymatic activity. However, patient A showed epileptic manifestations and microcephaly and her mother suffers from generalized tonic seizures, whereas the brother of the patient is clinically normal. The lack of correlation between genotype and phenotype in this family cannot easily be explained. However, both population screening based on DPD catalytic activity and a limited screening study for the common splice donor site mutation revealed that as much as 3–5% of the normal population could be heterozygotes for mutant DPD alleles (Lu et al. 1993; McMurry and McLeod 1996; Wei et al. 1996). These studies suggest that up to 1 in every 1000 births in the general population might be homozygous for DPD mutations. These figures are at least 10–20 times higher than the observed frequency for completely DPD-deficient patients, suggesting that either the vast majority of DPD-deficient individuals shows no clinical phenotype or that metabolic screening programs fail to detect these patients. Since, at least in the Netherlands, metabolic screening in pediatric patients with neurological symptoms always includes analysis of purines and pyrimidines, thus allowing the detection of DPD deficiency, it is more likely that indeed a number of patients show no characteristic clinical phenotype. Apparently, additional factors must play an important role in expression of the clinical phenotype. One of these factors might include β -alanine homeostasis since this neurotransmitter cannot be synthesized and can only be derived from dietary sources in DPD-deficient individuals (Van Kuilenburg et al. 1996). The relationship between β -alanine homeostasis and clinical symptoms is currently under investigation in our laboratory.

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