Dihydropyrimidinase Deficiency and Severe 5-Fluorouracil Toxicity

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ABSTRACT

Dihydropyrimidinase (DHP) is the second enzyme in the catabolism of 5-fluorouracil (5FU), and it has been suggested that patients with a deficiency of this enzyme are at risk from developing severe 5FU-associated toxicity. In this study, we demonstrated for the first time that in one patient the severe toxicity, after a treatment with 5FU, was attributable to a partial deficiency of DHP. Analysis of the DHP gene showed that the patient was heterozygous for the missense mutation 833G>A (G278D) in exon 5. Heterologous expression of the mutant enzyme in Escherichia coli showed that the G278D mutation leads to a mutant DHP enzyme without residual activity. An analysis for the presence of this mutation in 96 unrelated Dutch Caucasians indicates that the allele frequency in the normal population is <0.5%. Our results show that a partial DHP deficiency is a novel pharmacogenetic disorder associated with severe 5FU toxicity.

INTRODUCTION

5FU is one of the most commonly used chemotherapeutic agents for the systemic treatment of cancers of the gastrointestinal tract, breast, head, and neck. Recent advances in our understanding of the metabolism of 5FU, and the key enzymes involved in the activation and degradation of 5FU have led to an increased awareness that the catabolic route of 5FU plays an important role in the determination of toxicity as well as the efficacy toward 5FU (1–3). It has been reported that >80% of the administered 5FU is catalyzed by three consecutive enzymes of the pyrimidine degradation pathway (4).

DHP catalyzes the conversion of 5FU to FUH₂, which is the initial and rate-limiting step in this catabolism (Fig. 1). FUH₂ can be additionally degraded to fluoro-β-ureidopropionate and subsequently to fluoro-β-alanine by DHP and β-ureidopropionase, respectively. The pivotal role of DPD in chemotherapy using 5FU has been shown in cancer patients with a complete or partial deficiency of this enzyme. These patients suffered from severe toxicity, including death, after the administration of 5FU (5–8). A number of these patients proved to be heterozygous or homozygous for a mutant DPYD allele (5–9).

It has also been suggested that patients with a deficiency of DHP are at risk of developing severe 5FU-associated toxicity (10–12). However, no studies have been reported describing the analysis of the DHP gene for the presence of mutations in patients with severe 5FU toxicity. Here, we describe the first patient with severe 5FU-associated toxicity who proved to be heterozygous for a missense mutation in the DHP gene.

MATERIALS AND METHODS

Analysis of Dihydropyrimidines. The concentrations of dihydouracil and dihydrothymine in plasma were determined using reversed-phase HPLC combined with electrospray tandem mass spectrometry (13).

Determination of the DPD and DHP Activity. The activity of DPD in peripheral blood mononuclear cells was determined using a radiochemical assay with subsequent separation of radiolabelled thymine from radiolabelled dihydrothymine with reversed-phase HPLC (14). The activity of DHP was determined in an assay mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM DTT, and 500 μM [2-14C]-dihydouracil (1.85–2.22 GBq/mmol; Moravek Biochemicals, Brea, CA). Separation of radiolabelled dihydouracil from N-carbamyl-β-alanine was performed isocratically [50 mM NaH₂PO₄ (pH 4.5) at a flow rate of 1 ml/min] by reversed-phase HPLC on a Supelcosil LC-18-S column (250 × 4.6 mm; 5 μm particle size) with on-line detection of radioactivity (15). Protein concentrations were determined with a copper-reduction method using bichinchoninic acid, essentially as described by Smith et al. (16).

PCR Amplification of Coding Exons. DNA was isolated from granulocytes by standard procedures. The PCR amplification of all nine of the coding exons and flanking intronic regions was carried out using the primer sets as described previously (17). However, for exon 5, the sequence of the reverse primer was 5'-GGATCCAGATGGGAGGACGAC-3'. Forward primers had a 5'-TGTAAGAACGCGCCATGT-3' extension, whereas reverse primers had an 5'-CAGGAAAAAGCTATGACACG-3' extension at their 5'-ends. These sequences were complementary to the labeled -21M13 and M13 reversed primers used in the dye-primer sequence reaction. Amplification of all of the exons was carried out in 50-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50
mm KCl, 1.5–2.5 mm MgCl₂, 10 pmol of each primer, 200 µM of each dNTP, and 2 units of Taq polymerase (Promega Benelux B.V., Leiden, the Netherlands). After initial denaturation for 5 min at 96°C, amplification was carried out for 35 cycles (0.25 min 96°C, 0.5 min 60°C, and 0.5 min 72°C). PCR products were separated on 1% agarose gels, visualized with ethidium bromide, and purified using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany) or used for direct sequencing.

Expression of DHP in E. coli. An expression plasmid containing the wild-type human DHP cDNA (pSE420-DHP) was constructed by subcloning the EcoRI-BamHI insert of the cDNA encoding human DHP, into the pSE420 vector (18). The 833G>A mutation (G278D) was introduced into the human DHP sequence using the megaprimer technique and subsequently subcloned in a pSE420 vector (pSE420-DHP-G278D). The resulting clones were sequenced completely to verify the presence of the mutation and to exclude the presence of random mutations introduced by PCR artifacts. Expression plasmids were introduced into the E. coli strain BL21. A 5-ml Luria-Bertani broth culture, supplemented with 50 µg/ml ampicillin, was inoculated with 100 µl of an overnight preculture grown in Luria-Bertani broth. Cells were grown for 3 h at 37°C, and induction was performed by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Cells were sedimented after 3 h, washed with an isolation buffer [35 mm potassium phosphate (pH 7.4) and complete; Roche Diagnostics, Almere, the Netherlands] and resuspended in 500 µl of isolation buffer. The cell suspension was frozen for 16 h at −20°C, thawed on ice, and lysed by sonication. The crude lysate was centrifuged at 18,000 × g for 15 min, and the resulting supernatant was stored at −80°C.

Western Blot Analysis. Cell extracts (2.5 µg) were fractionated on a 7.5% (w/v) SDS-polyacrylamide gel and transferred to a nitrocellulose filter. Blocking of the membrane was performed for 16 h with TBS [25 mM Tris, 137 mM NaCl, and 2.7 mM KCl (pH 7.4)] containing 5% (w/v) nonfat dry milk. Subsequently, the membrane was incubated for 1 h with a 1:1000 dilution of rabbit antirat DHP polyclonal antibody in TBS, supplemented with 0.05% (v/v) Tween 20. The membranes were washed three times (5 min each) with TBS containing 0.05% (v/v) Tween 20 and incubated for 45 min with TBS containing 0.05% (v/v) Tween 20, 5% (w/v) nonfat dry milk, and a 1:5000 dilution of a pig antirabbit secondary antibody conjugated to horseradish peroxidase (Dako, Copenhagen, Denmark). After rinsing the membrane three times (5 min each) with TBS containing 0.05% (v/v) Tween 20, detection of DHP was performed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

DHPLC Analysis of the 833G>A Mutation. PCR fragments containing exon 5 were analyzed on a Agilent 1100-DHPLC system (Agilent Technologies Netherlands B.V., Amstelveen, the Netherlands). The buffer system for the DHPLC consisted of buffer A [0.1 M triethylammonium acetate and 1 mM EDTA (pH 7.0)] and buffer B [buffer A supplemented with 25% (v/v) acetonitrile]. Before injection, PCR fragments were denatured at 95°C followed by slow renaturation. PCR fragments (5 µl) were loaded in 40% buffer B on a Zorbax ds DNA column (Agilent Technologies Netherlands B.V.) and subsequently eluted, within 6 min at 65°C, with a gradient from 55–65% buffer B.

Sequence Analysis. Sequence analysis of genomic fragments amplified by PCR and expression plasmids was carried out on an Applied Biosystems model 3100 automated DNA sequencer using the dye-terminator and the dye-primer method (Perkin-Elmer Corp., Foster City, CA), respectively.

RESULTS

Clinical Evaluation. The patient was female, born in 1943, and had a history of an appendectomy, middle ear surgery, bilateral removal of cervical ribs, multiple cysts, and abscesses in the vagina and a hysterectomy. In addition, she suffered from obesity and diabetes mellitus. In 1991, the patient underwent a mastectomy for a ductal carcinoma (pT2N1M0) combined with five courses of CMF chemotherapy (60 mg methotrexate i.v., 900 mg 5FU i.v. on days 1 and 8, and p.o. 150 mg cyclophosphamide from days 1 to 14). The chemotherapy with CMF caused considerable mucosal toxicity and, therefore, the planned sixth course with CMF was cancelled. In 1994, the patient developed skeletal metastasis for which she was treated successively with tamoxifen, aminogluthetimide, and Re-186 etidronate. In 1997, lung metastasis was observed, and therapy was changed to chemotherapy with doxorubicin and cyclophosphamide. In April 1998, because of progression, she received radiotherapy and docetaxel. Because of disease progression,
Fig. 2 Genomic organization and conservation of amino acid sequences of the DHP gene. The DHP gene consists of 10 exons encoding an open reading frame of 1560 bp (top panel). The different mutations identified in patients with a deficiency of DHP are indicated, numbers correspond to the cDNA position. The bottom panel shows the alignment of the amino-acid sequences of DHP, DRP, and Hydantoinase (Hy) from Agrobacterium radiobacter (Ar), Arabidopsis thaliana (A), Bacillus stearothermophilus (Bs), bovine (b), Burkholderia pickettii (bp), Caenorhabditis elegans (Ce), Chicken (c), Dictyostelium discoideum (Dd), Drosophila melanogaster (Dm), Human (h), Mouse (m), Pseudomonas aeruginosa (Pa), Pseudomonas putida (Pp), Rat (r), Saccharomyces kluyveri (Sk), Streptomyces coelicolor (Sc), and Xenopus laevis (x). The GenBank accession numbers are depicted in parentheses. In Saccharomyces kluyveri, the inserted sequences were removed before alignment (24). Conserved amino-acid sequences are boxed, and the arrow indicates the position of the mutation G278D in human DHP.

palliative treatment commenced with continuous infusion of 5FU (300 mg/m²/day). During the first week of treatment she noticed increased pain while swallowing, and after 14 days, a skin rash was observed that developed to skin ulcers on the thorax, palms of the hands, and soles of both feet. Her condition worsened, and she became confused and was hospitalized with leucopenic fever and sepsis. At this stage, treatment with 5FU was aborted. Antibiotic treatment with floxapen and gentamicin neverthe less from severe 5FU associated-toxicity.

DPD Activity and Dihydropyrimidines in Plasma. A normal activity of DPD was detected in peripheral blood mononuclear cells of the patient (13.1 nmol/mg/h; controls 9.9 ± 2.8; n = 54), thus excluding a (partial) deficiency of DPD as the underlying cause of the severe toxicity, after the administration of 5FU. To investigate whether the severe toxicity might have been caused by a complete deficiency of DHP, plasma was collected to determine the levels of 5,6-dihydrouracil and 5,6-dihydrothymine. A normal concentration of 5,6-dihydrouracil (3.9 μM; controls 2.2 ± 3.1 μM; n = 97) and a high-normal concentration of 5,6-dihydrothymine (1.44 μM; controls 0.7 ± 0.4 μM; n = 97) were detected in plasma. The concentration of 5,6-dihydrothymine was at the upper limit of the 95% distribution range of the control population.

Sequence Analysis of the DHP Gene. Analysis of the genomic sequences of exons 1–9 of the DHP gene showed that the tumor patient was heterozygous for a missense mutation 833G>A in exon 5, leading to the amino acid substitution G278D (Fig. 2). In addition, the patient proved to be homozygous for a -1T>C mutation and heterozygous for a silent mutation 216C>T (F72F) in exon 1. No missense mutations could be detected in the DHP gene of 22 other patients, with normal DPD activity, but who suffered nevertheless from severe 5FU associated-toxicity.

Alignment of various eukaryotic DHP, DRP and prokaryotic (o-hydantoinase) protein sequences revealed that the gly cine at position 278 is conserved in DHP from mammals, Drosophila melanogaster, Dictyostelium discoideum, and Saccharomyces kluyveri, as well as in DRP1–4 (Fig. 2). In Caenorhabditis elegans and Arabidopsis thaliana, the glycine is replaced by a similar alanine and the hydrophobic residue isoleucine, respectively. However, in bacteria, significant sequence variation exists in the region around G278 when compared with DHP and DRP1–4 in mammals, and G278 was replaced by either hydrophobic or hydrophilic residues. This might indicate that significant structural differences exist around residue 278 in eukaryotic DHP and the hydantoinases from bacteria.

Expression Analysis of the G278D Mutation. To investigate the effect of the mutation G278D on the activity of DHP, the mutation was introduced into the pSE420-DHP vector by site-directed mutagenesis and expressed in E. coli. No endoge-
nous DHP activity (< 0.01 nmol/mg/h) could be detected in the E. coli strain used for the expression of the constructs. Introduction of the wild-type DHP construct increased the DHP activity >61,000-fold above the background. Expression of the DHP construct containing the G287D mutation yielded no detectable activity of DHP (Fig. 3).

To exclude the fact that the lack of enzyme activity was the result of an inability to produce the mutant protein in E. coli, we analyzed the expression level by immunoblotting. Fig. 3 shows that the mutant protein carrying the G287D mutation was expressed in comparable amounts as the wild-type protein. Furthermore, no DHP protein could be detected in mock-transfected cells. Thus, the lack of DHP activity in E. coli transfected with the pSE420-DHP-G278D construct is not because of rapid degradation of the mutant DHP protein in the E. coli lysates.

**Population Screening for the 833G>A Mutation.**

Screening of individuals for the presence of the 833G>A mutation was performed with DHPLC. Fig. 4 shows that there is a clear separation between heteroduplex DNA from homoduplex DNA. Therefore, distinctive chromatographic patterns were obtained for the presence of the 833G>A mutation. PCR fragments of the pSE420-DHP-G278D construct were analyzed on immunoblot with DHP-specific antibodies (right panel).

**DISCUSSION**

Although originally synthesized over 4 decades ago, 5FU remains one of the most commonly prescribed anticancer agents for the treatment of several common human malignancies, including those of the gastrointestinal tract, breast, head, and neck. Adverse drug reactions are a major clinical problem, and a meta-analysis involving 1219 patients with colorectal cancer showed that grade 3 to 4 toxicity was encountered in 31–34% of the patients receiving 5FU with 0.5% of the patients experiencing lethal toxicity (19). It is likely that a significant proportion of these adverse drug reactions are because of genetically based differences in the response to 5FU. In this respect, it has been shown that a (partial) deficiency of DPD is an important pharmacogenetic syndrome and responsible for the observed toxicity in 60% of the patients suffering from severe 5FU-associated toxicity (5, 8, 9). To date, the underlying mechanisms for the observed increased sensitivity toward 5FU, in patients with a normal DPD activity, is unknown.

To investigate the role of DHP, the second enzyme of the 5FU catabolic pathway, in the etiology of 5FU toxicity, we have analyzed the DHP gene in 23 tumor patients with normal DPD activity but, nevertheless, suffering from severe toxicity for the presence of mutations. One of these patients proved to be heterozygous for a 833G>A mutation in exon 5, and heterologous expression of this mutation showed that the mutant DHP protein bore no residual activity.

DHP deficiency is an autosomal recessive disease characterized by dihydropyrimidinuria and has been associated with a variable clinical phenotype (10–12, 17). Loading studies with uracil, in patients suffering from a complete DHP deficiency, showed strongly elevated levels and prolonged retention of uracil and dihydrouracil in serum, with >80% of the administered dose being excreted either unchanged or in the form of dihydrouracil during the first 24 h after the load (10, 11). In individuals heterozygous for a mutant DHP allele, the urinary concentration of dihydrouracil was several fold higher compared with that observed in controls, after loading with uracil (12). Under normal conditions, a low DHP activity is probably sufficient to maintain dihydrouracil and dihydrotymine homeostasis as heterozygotes do not excrete elevated levels of dihydropyrimidines. After the loading of such patients with uracil, the accumulation of dihydrouracil in urine increased several fold compared with normal individuals, indicating a decreased capacity of heterozygotes to degrade dihydropyrimidines (12). In this respect, it is worthwhile to note that the coadministration of FUH₂ with 5FU attenuated the antitumor activity and increased the toxicity of 5FU (20). Furthermore, in the presence of elevated concentrations of dihydropyrimidines, the reverse reaction, catalyzed by DPD, toward the pyrimidine bases is stimulated (10, 21). Thus, the decreased capacity to degrade FUH₂ and 5FU, because of a decreased DHP activity, might be directly responsible for the observed toxicity in the study patient. In this respect, it is worthwhile to note that neupronenia has also been associated with a partial deficiency of DPD (5, 7, 8).

The identification of genetic factors predisposing patients for development of severe 5FU-associated toxicity is increas-
ingly being recognized as an important field of study. It has been shown recently that the C677T polymorphism in the methylenetetrahydrofolate reductase gene might be responsible for the severe toxicity observed in some breast cancer patients receiving adjuvant treatment with cyclophosphamide, methotrexate, and 5FU (22). In addition, it has been suggested that a polymorphism in the enhancer region of the thymidylate synthase gene promoter is associated with toxicity toward 5FU (23).

Our results indicate that a partial DHP deficiency is not a major determinant in the etiology of 5FU toxicity. To date, only 9 individuals suffering from a complete DHP deficiency have been reported, which, to some extent, may be because of the lack of specific and efficient methods in most laboratories to detect the dihydropyrimidinases. In fact, the prevalence of a DHP deficiency in Japan has been estimated to be 1 in 10,000, which is comparable with the estimated frequency of patients with a DPD deficiency in the Netherlands (6). Therefore, a DHP deficiency might be less rare than generally assumed.

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