Lethal Outcome of a Patient with a Complete Dihydropyrimidine Dehydrogenase (DPD) Deficiency after Administration of 5-Fluorouracil: Frequency of the Common IVS14+1G>A Mutation Causing DPD Deficiency

INTRODUCTION

5FU remains one of the most widely used chemotherapeutic agents for the systemic treatment of cancers of the gastrointestinal tract, breast, and head and neck. As a single drug, 5FU has only limited efficacy. To improve the clinical response of 5FU, optimal administration schedules as well as the combination of 5FU with other drugs, which should increase its antitumor activity or decrease the host toxicity, have been investigated. It has been shown that long-term continuous i.v. infusion of 5FU is superior to bolus injections of 5FU in terms of response rate, however, only a small increase in median survival was observed (1), and high inter- and intrapatient variations in the plasma concentrations of 5FU have been observed during prolonged infusion of the drug (2). In addition, a relationship between the 5FU dose intensity and the therapeutic response, as well as toxicity, has been noted (3, 4).

An important determinant in predicting the toxicity as well as the efficacy of 5FU might be the activity of DPD. DPD is the initial and rate-limiting enzyme in the catabolism of 5-fluorouracil (5FU), and it is suggested that patients with a partial deficiency of this enzyme are at risk from developing a severe 5FU-associated toxicity. In this study, we demonstrated that a lethal toxicity after a treatment with 5FU was attributable to a complete deficiency of DPD. Analysis of the DPD gene for the presence of mutations showed that the patient was homozygous for a G→A mutation in the invariant GT splice donor site flanking exon 14 (IVS14+1G>A). As a consequence, no significant residual activity of DPD was detected in peripheral blood mononuclear cells. To determine the frequency of the IVS14+1G>A mutation in the Dutch population, we developed a novel PCR-based method allowing the rapid analysis of the IVS14+1G>A mutation by RFLP. Screening for the presence of this mutation in 1357 Caucasians showed an allele frequency of 0.91%. In our view, the apparently high prevalence of the IVS14+1G>A mutation in the normal population, with 1.8% heterozygotes, warrants genetic screening for the presence of this mutation in cancer patients before the administration of 5FU.

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The abbreviations used are: 5FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; PBM, peripheral blood mononuclear.
molecular mechanisms underlying the 5FU-induced death been resolved. In this paper, we describe a simple genotyping procedure to test for the presence of the IVS14+1G>A mutation. Furthermore, we describe the first patient with lethal toxicity, after the administration of 5FU, who proved to be homozygous for the IVS14+1G>A mutation.

**MATERIALS AND METHODS**

**Chemicals.** [4-14C]-thymine (1.85–2.22 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). Lymphoprep (specific gravity, 1.077 g/ml; 280 mOsm) was obtained from Nycomed Pharma AS (Oslo, Norway). LeucoSep tubes were supplied by Greiner (Frickenhausen, Germany). HAM-F10 medium with 20 mM HEPES was obtained from Life Technologies, Inc. (Breda, the Netherlands). AmpliTaq Taq polymerase BigDye-Terminator-Cycle-Sequencing-Ready Reaction kits were supplied by Perkin-Elmer Corp. (Foster City, CA). Restriction endonuclease *NdeI* was obtained from Roche Diagnostics Nederland B.V. (Almere, the Netherlands). A Qiaquick Gel Extraction kit was obtained from Qiagen (Hilden, Germany). All other chemicals used were of analytical grade.

**Analysis of Pyrimidine Bases.** The concentrations of the pyrimidine bases, uracil and thymine in plasma, were determined using reversed-phase HPLC combined with diode-array detection, as described before (16).

**Culture Conditions of Human Fibroblasts.** Fibroblasts were cultured from skin biopsies obtained from controls and the index patient. Biopsies were incubated at 37°C in HAM-F10 medium, supplemented with 20 mM HEPES and 1% (v/v) FCS in 25-cm² cell-culture flasks until an adequate number of proliferating cells was obtained. Subsequently, cells were cultured in HAM-F10 medium supplemented with 20 mM HEPES and 10% (v/v) FCS. Fibroblasts were harvested with 0.25% (w/v) trypsin, and after washing the cells once with PBS and twice with 0.9% (w/v) NaCl, the cells were collected by centrifugation (175 × g at 7°C for 5 min), and the supernatant was discarded. The pellets were stored at −80°C.

**Isolation of Human PBM Cells and Granulocytes.** PBM cells were isolated from 15 ml EDTA-anticoagulated blood by centrifugation over Lymphoprep, and the cells from the interface were collected and treated with ice-cold NH₄Cl to lyse the contaminating erythrocytes, as described before (17). The pellet of the centrifugation step over Lymphoprep containing the granulocytes and erythrocytes was diluted with 7 ml of supplemented PBS [9.2 mM Na₂HPO₄, 1.3 mM NaH₂PO₄, 140 mM NaCl, 0.2% (w/v) BSA, 13 mM sodium citrate, and 5 mM glucose (pH 74)] and centrifuged at 800 × g at room temperature for 10 min. To lyse the erythrocytes, the pellet was resuspended in 7 ml of ice-cold ammonium chloride solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) and kept on ice for 5 min. After the addition of 10 ml of ice-cold supplemented PBS, the solution was centrifuged at 250 × g at 4°C for 10 min. The pellet was collected and subjected to another lysis step as described above. The pellet containing the granulocytes was washed once more with supplemented PBS, and the final cell pellet was frozen in liquid nitrogen and stored at −80°C until further analysis.

**Determination of the DPD Activity.** The activity of DPD was determined in a reaction mixture containing 35 mM potassium phosphate (pH 7.4), 2.5 mM MgCl₂, 1 mM DTT, 250 μM NADPH, and 25 μM [4-14C]-thymine (17). Separation of radiolabeled thymine from radiolabeled dihydrothymine was performed isocratically [50 mM NaH₂PO₄ (pH 4.5) at a flow rate of 2 ml/min] by high-performance liquid chromatography on a reversed-phase column (Alltima C18; 250 × 4.6 mm; 5-μm particle size; Alltech Associates Inc., Deerfield, IL) and protected by a guard column (Supelguard LC-18-S; 5-μm particle size; 20 × 4.6 mm; Supelco, Bellefonte, PA) with online detection of the radioactivity, as described before (17). Protein concentrations were determined with a copper-reduction method using bicinchoninic acid, essentially as described by Smith et al. (18).

**PCR Amplification of Exon 14.** DNA was isolated from purified granulocytes by standard procedures. PCR amplification of exon 14 and its flanking intronic regions was carried out using the primer sets DPD14a and DPD14, as specified in Table 1. Amplification of exon 14 was carried out in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 10 pmol each primer, 200 μM each deoxynucleotide triphosphate, and 2 units of Taq polymerase. After initial denaturation for 5 min at 95°C, amplification was carried out for 35 cycles (1 min 95°C, 1 min 55°C, 1 min 72°C). The PCR product was separated on 1% agarose gels, visualized with ethidium bromide, and purified using a Qiaquick Gel Extraction kit or used for direct sequencing.

**RFLP of Exon 14.** PCR amplification of exon 14 and its flanking 5′ donor intronic region was carried out using the primer sets NDE1 and NDE2, as specified in Table 1. Amplification of exon 14 was carried out in a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 5 pmol each primer, 200 μM each deoxynucleotide triphosphate and 2 units of Taq polymerase. After initial denaturation for 5 min at 96°C, amplification was carried out for 35 cycles (0.5

### Table 1 Oligonucleotides used for genomic PCR of exon 14 of the DPD gene

| Primer | Sequence | Direction | Position
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>DPD14a</td>
<td>5′-GATGCCTGCCTATGGGAAGGGACT-3′</td>
<td>Sensese</td>
<td>451–477</td>
</tr>
<tr>
<td>DPD14a</td>
<td>5′-GCTGCTCCTGGCTGGGTGAGGATT-3′</td>
<td>Antisense</td>
<td>762–783</td>
</tr>
<tr>
<td>NDE1</td>
<td>5′-ATCAGGACATTGGACATATGTTTC-3′</td>
<td>Sensese</td>
<td>565–589</td>
</tr>
<tr>
<td>NDE2</td>
<td>5′-CGTTTTTATGATTAAAATCAT-3′</td>
<td>Antisense</td>
<td>736–762</td>
</tr>
</tbody>
</table>

*a* Numbering according to the intron sequences flanking exon 14 of *DPYD* as published by Vreken et al. (19).

*b* The *NdeI* restriction sites introduced by site-directed mutagenesis (primers NDE1 and NDE2) are underlined. The single-base mismatches introducing the *NdeI* restriction site are depicted in boldface.
min 96°C, 0.5 min 60°C, and 1 min 72°C). Restriction analysis of the PCR products was performed in a 20-μl reaction mixture containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithioerythritol, 1 μl of PCR product, and 10 units of restriction endonuclease DpnI. The mixture was incubated overnight at 37°C. The DNA fragments were subsequently separated on a 3% agarose gel and visualized with ethidium bromide. 

**Sequence Analysis.** Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems model 377 automated DNA sequencer using the dye-terminator method.

**RESULTS**

**Clinical Evaluation.** The patient was a female 44 years of age. At the age of 40, she underwent a curative resection of a Dukes B2 moderately differentiated rectum carcinoma and subsequent adjuvant radiotherapy. Three years later, the patient presented with an ileus caused by an intra-abdominal recurrence of the tumor at the right hypochondrium. Debunkling of the tumor was performed as well as a partial ileal resection and extirpation of the uterus including the adnexes. No measurable lesions were present after the operation. After a period of 11 months, a palpable mass was present in the right lower abdomen and palliative chemotherapy was started with 5FU (900 mg) and leucovorin (200 mg), which was administered i.v. on a weekly basis. Five days after the first treatment, the patient developed stomatitis. Five days after the second injection with 5FU, the patient suffered from fever, severe stomatitis, leucopenia (WBCs, 1.6 × 10⁹/l) and thrombocytopenia (42 × 10⁹/l). The next day, the patient developed a severe pancytopenia for which she received a transfusion with erythrocytes and thrombocytes. Physical examination revealed that the palpable mass in her abdomen had greatly decreased. Despite intensive medical care, the patient died 8 days later because of infectious complications.

**Uracil and Thymine Levels in Plasma.** To investigate whether the lethal toxicity after the administration of 5FU might have been caused by a partial or complete deficiency of DPD, plasma was collected for determination of the levels of uracil and thymine. Strongly elevated concentrations of uracil (80 ± 0.2 nmol/mg/h; n = 20) and thymine (17.5 ± 0.2 nmol/mg/h; n = 20) were detected in plasma which is indicative of a complete deficiency of DPD.

**DPD Activity in PBM Cells and Fibroblasts.** DPD activity was determined in PBM cells isolated from a blood sample obtained during pancytopenia. Morphological examination of the isolated PBM cells on a cytospin preparation showed the presence of only lymphocytes and some residual thrombocytes. Hardly any activity of DPD (0.09 nmol/mg/h) was detected in the PBM cells of the patient who compared with that observed in controls (10.0 ± 3.4 nmol/mg/h; n = 22). Surprisingly, a low but significant activity of DPD (0.56 nmol/mg/h) was detected in thrombocytes when compared with controls (1.7 ± 0.6 nmol/mg/h; n = 22). Because the patient received thrombocyte transfusions, we feel that the very low DPD activity detected in PBM cells of the patient is most likely attributable to contamination of the PBM cell fraction by thrombocytes of the donor. No activity of DPD (<0.009 nmol/mg/h; controls, 0.89 ± 0.56 nmol/mg/h; n = 21) could be detected in cultured fibroblasts established from a skin biopsy of the patient.

**Sequence Analysis of the DPD Gene.** To investigate whether or not the common IVS14+1G>A mutation in the DPD gene might underlie the complete DPD deficiency in the tumor patient, two intron-specific primers were used for amplification of exon 14 and its flanking intron sequences (19). Sequence analysis showed that the tumor patient was homozygous for the IVS14+1G>A mutation, which changes the invariant splice donor site from GT to AT.

**RFLP Analysis of the IVS14+1G>A Mutation.** A PCR primer with a single-base mismatch was used, which introduced a NdeI restriction site in case a G→A point mutation is present in the invariant splice donor site of exon 14, to rapidly screen for the IVS14+1G>A mutation by RFLP (Fig. 1). As a positive control for the digestion efficiency of NdeI, an additional NdeI restriction site was introduced in the 5’ region of the PCR fragment using a forward PCR primer containing an A→T mismatch (Table 1). Under these conditions, the undigested PCR fragment has a length of 198 bp. After digestion with NdeI, the wild-type allele will produce two fragments of 181 bp and 17 bp. In contrast, the PCR fragment containing the G→A point mutation in the invariant splice donor site of exon 14 will produce three fragments of 17 bp, 154 bp, and 27 bp after digestion with NdeI. A diagrammatic representation of the PCR amplification of exon 14 and the subsequent analysis of the IVS14+1G>A mutation by RFLP is shown in Fig. 1. The validity of the concept is demonstrated by analysis of the DPDYD gene of a patient homozygous for the IVS14+1G>A mutation, an individual who is obligate heterozygous for the IVS14+1G>A mutation and the wild-type DPDYD gene from a control (Fig. 2).

Through analysis of 1357 Dutch Caucasians for the presence of the IVS14+1G>A mutation, we identified 24 individuals who were heterozygous for this mutation. Thus, the frequency of heterozygotes in the Dutch population is 1.8%. No individuals were detected who were homozygous for the IVS14+1G>A mutation, resulting in an allele frequency of 0.91%.

**DISCUSSION**

Because 5FU has a relatively narrow therapeutic index, toxicity increases as the dose is increased, resulting in escalated plasma levels of the drug (3, 4). Although the cytotoxic effects
A mutation in the invariant splice donor site is by far the most common one (52%) among patients with a complete deficiency of DPD (13). Furthermore, there appears to be some kind of homogeneity for the IVS14+1G>A mutation in Northern Europe (13). To date, the frequency of this mutation in the population has not been thoroughly investigated. Screening for the presence of the G→A splice site mutation in only a very limited number of individuals has revealed heterozygosity for this mutation in 1% of the Finnish population (180 alleles analyzed) and none in the British (60 alleles), Japanese (100 alleles), African-American (210 alleles), or Dutch (100 alleles) populations (19, 24). These analyses of the presence of the G→A splice site mutation were performed using RFLP, based on the fact that the G→A point mutation destroys a unique MaelI restriction site, present in the amplified genomic fragment encompassing the skipped exon and its flanking sequences (9, 19). Unfortunately, the restriction enzyme MaelI is rather expensive and thus not suitable for use in screening large numbers of individuals on a routine basis. Recently, a RFLP procedure has been developed for the detection of the IVS14+1G>A mutation using a SnaBI restriction site, which was introduced using PCR-mediated site-directed mutagenesis (25). A serious drawback of this method, however, is the fact that the SnaBI site will not be present in the homozygous-deficient type, whereas no positive control for the cutting efficiency of the restriction enzyme was present. For these reasons, we have developed a novel genotyping test for the IVS14+1G>A mutation. In our test, a Ndel restriction site would be introduced, with PCR-mediated site-directed mutagenesis, in the invariant GT splice donor site in the intron downstream of exon 14, in the event that a G→A point mutation is present. As a positive control for the restriction efficiency of Ndel, a second Ndel restriction site was introduced in the 3′ region of the amplified genomic fragment.

Using this genotyping test for the G→A mutation, we demonstrated that there is a relatively high frequency of the mutated allele in the normal Dutch population, with an allele frequency of 0.91%. Using the Hardy-Weinberg equilibrium and a frequency of heterozygotes of 1.8%, one can estimate the number of individuals homozygous for the G→A mutation to be 1.2 in 10,000. Compared with other frequently occurring inborn errors of metabolism, such as phenylketonuria (1:20,000), the expected prevalence of a DPD deficiency is high. Considering the common use of 5FU in the treatment of cancer patients and the increasing percentage of patients receiving high doses of 5FU in adjuvant therapy, the apparently high prevalence of the IVS14+1G>A mutation in the normal population warrants genetic screening for the presence of this mutation in cancer patients before the administration of 5FU. In this way, the serious and sometimes lethal 5FU-related toxicities encountered in patients heterozygous or homozygous for the IVS14+1G>A mutation might be prevented.
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