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van Kuilenburg, A.B.P.; Vreken, P.; Meinsma, R.; van Lenthe, H.; Beex, L.V.A.M.; de Abreu, R.A.; van Gennip, A.H.

**DOI**

[10.1016/S0959-8049\(97\)00261-X](https://doi.org/10.1016/S0959-8049(97)00261-X)

**Publication date**

1997

**Published in**

European Journal of Cancer

[Link to publication](#)

**Citation for published version (APA):**

van Kuilenburg, A. B. P., Vreken, P., Meinsma, R., van Lenthe, H., Beex, L. V. A. M., de Abreu, R. A., & van Gennip, A. H. (1997). Heterozygosity for a point mutation in an invariant splice donor site of dihydropyrimidine dehydrogenase and severe 5-fluorouracil related toxicity. *European Journal of Cancer*, 33, 2258-2264. [https://doi.org/10.1016/S0959-8049\(97\)00261-X](https://doi.org/10.1016/S0959-8049(97)00261-X)

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PII: S0959-8049(97)00261-X

## Original Paper

# Heterozygosity for a Point Mutation in an Invariant Splice Donor Site of Dihydropyrimidine Dehydrogenase and Severe 5-Fluorouracil Related Toxicity

A.B.P. Van Kuilenburg,<sup>1</sup> P. Vreken,<sup>1</sup> L.V.A.M. Beex,<sup>2</sup> R. Meinsma,<sup>1</sup> H. Van Lenthe,<sup>1</sup>  
R.A. De Abreu<sup>3</sup> and A.H. Van Gennip<sup>1</sup>

<sup>1</sup>Academic Medical Center, Divisions of Clinical Chemistry and Pediatrics, Fo-224, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam; <sup>2</sup>Academic Hospital Nijmegen, Department of Endocrine Diseases, Nijmegen; and <sup>3</sup>Academic Hospital Nijmegen, Department of Pediatrics, Nijmegen, The Netherlands

Dihydropyrimidine dehydrogenase (DPD) is responsible for the breakdown of the widely used anti-neoplastic agent 5-fluorouracil (5-FU), thereby limiting the efficacy of the therapy. It has been suggested that patients suffering from 5-FU toxicities due to a low activity of DPD are genotypically heterozygous for a mutant allele of the gene encoding DPD. In this study we investigated the cDNA and a genomic region of the DPD gene of a cancer patient experiencing severe toxicity following 5-FU treatment for the presence of mutations. Although normal activity of DPD was observed in fibroblasts, the DPD activity in leucocytes of the cancer patient proved to be in the heterozygous range. Analysis of the DPD cDNA showed heterozygosity for a 165 bp deletion that results from exon skipping. Sequence analysis of the genomic region encompassing the skipped exon showed that the tumour patient was heterozygous for a G→A point mutation in the invariant GT splice donor sequence in the intron downstream of the skipped exon. So far, the G→A point mutation has also been found in 8 out of 11 patients suffering from a complete deficiency of DPD. Considering the frequent use of 5-FU in the treatment of cancer patients, the severe 5-FU-related toxicities in patients with a low activity of DPD and the high frequency of the G→A mutation in DPD deficient patients, analysis of the DPD activity and screening for the G→A mutation should be routinely carried out prior to the start of the treatment with 5-FU. © 1997 Elsevier Science Ltd.

**Key words:** dihydropyrimidine dehydrogenase, 5-fluorouracil, exon skipping, cardiotoxicity, hyperpigmentation

*Eur J Cancer*, Vol. 33, No. 13, pp. 2258-2264, 1997

## INTRODUCTION

DIHYDROPYRIMIDINE DEHYDROGENASE (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases and it catalyses the reduction of thymine and uracil to 5,6-dihydrothymine and 5,6-dihydrouracil, respectively. In children, the deficiency of DPD is often accompanied by a neurological disorder but a considerable variation in the clinical presentation among these patients has been reported [1]. In these patients, a large accumulation of uracil and thymine has been detected in urine, blood and in

cerebrospinal fluid [1-3]. Recently, we provided the first evidence at the molecular level that the mRNA of a patient with a deficiency of dihydropyrimidine dehydrogenase lacked a segment of 165 nucleotides that results from exon skipping with the splicing error being caused by a point mutation in an invariant GT splice donor sequence in the intron downstream of the skipped exon [4-6].

DPD is also responsible for the breakdown of the widely used antineoplastic agent 5-fluorouracil (5-FU), thereby limiting the efficacy of the therapy. 5-FU is one of the few drugs that shows some antitumour activity against various otherwise untreatable tumours including carcinomas of the gastrointestinal tract, breast, ovary and skin. Although the

Correspondence to A.B.P. Van Kuilenburg.

Received 16 Oct. 1996; revised 17 Feb. 1997; accepted 17 Mar. 1997.

cytotoxic effects of 5-FU are probably directly mediated by the anabolic pathways, the catabolic route plays a significant role since more than 80% of the administered 5-FU is catabolised by DPD [7]. Indeed, inhibitors of DPD have been shown to potentiate the effect of 5-FU *in vitro* and *in vivo* [8–10].

The important role of DPD in chemotherapy with 5-FU has been shown in cancer patients with a complete or near-complete deficiency of this enzyme. These patients suffered from severe (neuro)toxicity, including death, following 5-FU chemotherapy [11–19], including 2 cancer patients with a complete deficiency of DPD [12, 13], 3 patients with a suspected deficiency of DPD [11, 15] and 13 patients with a very low activity of DPD [14, 16–19]. A clinical pharmacological study of one of these patients with a complete deficiency of DPD demonstrated minimal catabolism of 5-FU with a 10-fold longer 5-FU half-life compared with patients with a normal activity of DPD [12].

It has been suggested that patients suffering from 5-FU toxicities due to a low activity of DPD are genotypically heterozygous for a mutant allele of the gene encoding DPD [20]. Furthermore, the frequency of heterozygotes in the normal population has been estimated to be as high as 3% [20–23]. So far, heterozygosity for a mutated DPD allele has only been shown in 1 cancer patient [24]. In this paper, we demonstrate that a patient experiencing severe toxicity following 5-FU chemotherapy proved to be heterozygous for a G→A point mutation in an invariant GT splice donor sequence, leading to faulty splicing. Furthermore, the G→A point mutation has also been found in 8 out of 11 other patients suffering from a complete DPD deficiency.

## MATERIALS AND METHODS

### Materials

AmpliTaQ Taq polymerase was supplied by Perkin–Elmer (San Jose, California, U.S.A.). A Qiaquickspin PCR purification kit was obtained from Qiagen (Hilden, Germany). Dye-primer cycle-sequence-ready reaction kits were obtained from Applied Biosystems (San Jose, California, U.S.A.). Oligonucleotides were synthesised on a Millipore Expedite oligonucleotide synthesiser. HAM-F10 culture medium, fetal calf serum and newborn calf serum were obtained from Gibco Laboratories (Paisley, Scotland). [2-<sup>14</sup>C]Thymine was obtained from Biotrend GmbH (Köln, Germany). Lymphopaque was obtained from Nyegaard & Co (Oslo, Norway). LeucoSep tubes were supplied by Greiner (Frickenhausen, Germany). All other chemicals were of analytical grade.

### Culture conditions of the human fibroblasts

Fibroblasts were cultured from skin biopsies obtained from controls (healthy volunteers and patients admitted to our hospital with clinical and biochemical findings not indicative of inborn errors in the purine and pyrimidine metabolism, mitochondrial oxidative phosphorylation or disorders in the urea cycle), patients with a DPD deficiency and obligate heterozygotes (e.g. parents of the affected family member).

Biopsies were incubated at 37°C in HAM-F10 medium supplemented with 20 mM HEPES and 20% (v/v) fetal calf serum in 25 cm<sup>2</sup> cell-culture flasks until an adequate number of proliferating cells was obtained. Subsequently, the cells were harvested with 0.25% (w/v) trypsin and distributed over two 75 cm<sup>2</sup> flasks. These cells were cultured in HAM-F10 medium supplemented with 20 mM HEPES and 15% (v/v)

newborn calf serum. Monolayers of fibroblasts were grown to confluency and harvested with 0.25% (w/v) trypsin. After washing the cells twice with PBS (9.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.3 mM NaH<sub>2</sub>PO<sub>4</sub> and 140 mM NaCl, pH 7.4) and once with 0.9% (w/v) NaCl, the cells were collected by centrifugation (175 g, for 5 min) and the supernatant was discarded. The pellets were stored at –80°C.

### Isolation of human leucocytes

Leucocytes were isolated from 15 ml EDTA-anticoagulated blood using lymphopaque (spec. gravity 1.086 g/ml, 350 mOsm). The blood sample (± 7 ml) was layered on 3 ml of lymphopaque using 10 ml LeucoSep tubes and centrifuged at 800 g at room temperature for 20 min. The interface containing the leucocytes was collected, diluted with phosphate-buffered saline (PBS; 9.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.3 mM NaH<sub>2</sub>PO<sub>4</sub> and 140 mM NaCl, pH 7.4) to a final volume of approximately 12 ml and centrifuged at 800 g for 8 min. To lyse the erythrocytes, the pellet was resuspended in 5 ml ice-cold ammonium chloride solution (8.29 g/l NH<sub>4</sub>Cl, 1.00 g/l KHCO<sub>3</sub> and 37.2 mg/l EDTA) and kept on ice for 5 min. After the addition of 7 ml ice-cold PBS, the solution was centrifuged at 250 g at 4°C for 8 min. This step was repeated twice to remove the platelets. The resulting pellet was resuspended in 3 ml PBS and an aliquot was used for cell counting and for a differential count to determine the purity of the leucocyte pellet. The remaining suspension was centrifuged at 11 000 g at 4°C for 10 s. The supernatant was discarded and the pellet was frozen in liquid nitrogen and stored at –80°C until further analysis.

### Preparation of fibroblast and leucocyte homogenates

The frozen cell pellets of fibroblasts and leucocytes were suspended in 300 µl 35 mM potassium phosphate (pH 7.4) and 2.5 mM MgCl<sub>2</sub> and sonicated three times at 4 W (Vibra-cell Sonicator, output control 20%) for 10 s with intervals of 30 s under constant cooling in ice-water. After centrifugation (11 000 g at 4°C for 20 min), the supernatants were removed and saved for further analysis at –80°C. Protein concentrations in the supernatants were determined with a copper-reduction method using bicinchoninic acid, essentially as described by Smith and associates [25].

### Determination of dihydropyrimidine dehydrogenase activity

The activity of dihydropyrimidine dehydrogenase was determined in a reaction mixture containing 35 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol, 2.5 mM MgCl<sub>2</sub>, 250 µM NADPH and 25 µM [<sup>14</sup>C] thymine. Separation of radiolabelled thymine and the reaction product dihydrothymine was performed isocratically (50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 4.5) at a flow rate of 2 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 [26].

### Isolation of RNA and DNA

RNA and DNA were isolated from cultured fibroblasts by the guanidinium thiocyanate method [27] and standard procedures [28], respectively.

### PCR analysis

cDNA synthesis and RT-PCR reactions for amplifying cDNA coding for DPD were carried out essentially as described before [4, 6]. Four overlapping DPD cDNA

fragments were amplified using primer sets as specified in Table 1. Forward primers had an 5'-TGTA AAC-GACGGCCAGT-3' extension, whereas reverse primers had an 5'-CAGGAAACAGCTATGACC-3' extension at their 5'-ends. These sequences are complementary to the labelled M13 and M13 reversed primers used in the dye-primer sequence reaction. Amplification was carried out in 50 µl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 1 µM of each primer and 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).

Amplification of the genomic region containing a DPD exon and its flanking sequences was carried out essentially as described before with the primers I<sub>1</sub> and I<sub>2</sub> [6]. These primers also contained the above-mentioned 5'-extensions for sequencing purposes. PCR products were separated on 1% agarose gels, visualised with ethidium bromide and purified using a Qiaquickspin PCR purification kit and used for direct sequencing.

#### Sequence analysis

Sequence analysis was carried out on a Applied Biosystems model 377 automated DNA sequencer using dye-primer method for the DPD cDNA and genomic fragments.

#### Statistical analysis

The differences between the mean activity of DPD in fibroblasts of controls and obligate heterozygotes were analysed with the two-sample *t*-test using log transformed data. Similar results were obtained with analysis by the non-parametric Mann-Whitney test of the original data. The level of significance was set a priori at  $P \leq 0.05$ . Analyses were performed with the Statistical Package for the Social Sciences (SPSS Inc. Chicago, Illinois, U.S.A.).

## RESULTS

#### Clinical evaluation

The patient is a female (4-3-1931) who was treated for hyperthyroidism (Graves' disease) with thyrostatics and radioactive iodine in 1981. Subsequently she developed a hypothyroidism for which she was treated with L-thyroxine. She was known to be hypertensive and the treatment

modality consisted of protracted use of propranolol and dytenzide.

In December 1989, a mastectomy was performed due to an infiltrative ductal carcinoma in her right breast which was partly of the lobular type and manifested as Paget's disease. Postoperative staging showed pT4 with metastases in two of the 19 removed axillary lymph nodes with extranodal growth. The patient received radiation on the thorax, axillary lymph nodes and supraclavicular lymph nodes. Investigation of the tumour tissue showed the presence of oestrogen and progesterone receptors. After local and regional treatment, adjuvant chemotherapy started on 9 April 1990, with tamoxifen (20 mg) to be administered twice daily for 2 years, 3-amino-1-hydropropylidene bisphosphonate (150 mg) twice a day and 5-FU (15 mg/kg) to be administered intravenously every week. The first two injections with 900 mg 5-FU were tolerated well by the patient without complications. However, one day after the third intravenous injection with 5-FU she developed a serious diarrhoea without blood and suffered from nausea and vomiting. Physical examination showed dehydration and a diffuse sensitive abdomen, whereas no abnormalities of the mouth and throat were observed. Furthermore, the liver and spleen were not enlarged. She was slightly hypotensive (110/70 mmHg) accompanied with an increased pulse rate. Nine days after admission of the third 5-FU injection, severe hypokalaemia (2.1 mmol/l), hyponatraemia (127 mmol/l) and leucopenia ( $1.6 \times 10^9/l$ ) with a normal number of thrombocytes ( $199 \times 10^9/l$ ) was observed. The next day, the number of leucocytes and thrombocytes decreased further to  $0.9 \times 10^9/l$  and  $162 \times 10^9/l$ , respectively. During this period the patient developed fever (39°C). Since examination of urine, stools and sputum revealed no indication for infectious agents, treatment consisted of administration of liquid and supplementation of minerals. Her body temperature fell spontaneously and therefore no antibiotics were administered. During the subsequent days the clinical picture and haematological parameters gradually improved and normalised.

A conspicuous pigmentation of the operation scar and lines of the hands was also observed which disappeared during subsequent weeks. Furthermore, it should be noted that ECGs made on 26 April and 2 May 1990, showed extreme T wave inversions reflecting 5-FU-associated cardiotoxicity

Table 1. Oligonucleotides for DPD genomic and RT-PCR

Primer	Sequence 5' 3'	Direction	Position
A <sub>f</sub>	CTCGAGACTGTAGGCACTGCCA	Sense	61-82*
A <sub>r</sub>	TCATACGGCAGCCGGAAGTGGG	Antisense	775-797*
B <sub>f</sub>	TGCTTCCTTTTTGGCTCGATTGGG	Sense	687-710*
B <sub>r</sub>	CGGCCATTTCTACACTAATGTCCACC	Antisense	1671-1696*
C <sub>f</sub>	ATCGGTGAATGATGGAAAGCAAGC	Sense	1554-1577*
C <sub>r</sub>	AGCCAAAATGGGAAATCCAGGCAG	Antisense	2434-2457*
D <sub>f</sub>	CCAGCAGTGGGGATTGCAAAGC	Sense	2335-2356*
D <sub>r</sub>	TTGGAAAGAGCTGAACACAAGGATC	Antisense	3226-3250*
I <sub>1</sub>	TCCTCTGCAAAAATGTGAGAAGGGACC	Sense	451-477†
I <sub>2</sub>	TCACCAACTTATGCCAATTCTC	Antisense	762-783†

\*Numbering according to the DPD cDNA sequence as published by Yokota and associates [29]. †Numbering according to the intron sequences flanking the DPD exon as published by Vreken and associates [6].

when compared to ECGs made in October 1989 and May 1991. The observed cardiotoxicity reflects an increased coronary insufficiency in this patient who is familiar with coronary artery disease for which she underwent a by-pass operation in 1986.

Due to the excessive toxicity following the administration of 2700 mg 5-FU in 2 weeks, no further treatment with 5-FU was administered. The treatment with tamoxifen was continued up to 9 April 1992. Up to now, the patient is in good health with no evidence of recurrent cancer.

To investigate whether the excessive toxicity following the administration of 5-FU might have been caused by a complete deficiency of DPD, urine was collected on 9 May 1990. However, no abnormalities were observed in the concentration of uracil and thymine.

#### Activity of DPD in leucocytes and fibroblasts

To investigate whether the severe fluorouracil toxicity in this patient might have been caused by a near-complete deficiency of DPD, we determined the activity of DPD in leucocytes and in cultured fibroblasts established from a skin biopsy. Figure 1 shows that the mean activity of DPD in fibroblasts of controls of  $0.89 (\pm 0.56, \text{S.D.})$  nmol/mg/h is significantly higher ( $P=0.002$ ) than that of  $0.26 (\pm 0.20, \text{S.D.})$  nmol/mg/h observed in fibroblasts of obligate heterozygotes. In patients suffering from a complete deficiency of DPD, the activity proved to be undetectably low. Surprisingly, the activity of DPD in fibroblasts of the tumour patient (1.1 nmol/mg/h) was within the range of the controls and above the upper limit of the range of DPD activities as observed for the obligate heterozygotes (0.06–0.66 nmol/mg/h). In contrast, in leucocytes the activity of DPD of the tumour patient (3.1 nmol/mg/h) was decreased compared to the mean activity of DPD of controls ( $9.6 \text{ nmol} \pm 3.7 \text{ nmol/mg/h}$ ) and was comparable to that observed for obligate heterozygotes (Figure 2).

#### RT-PCR analysis of DPD mRNA in cultured fibroblasts

Total RNA isolated from fibroblasts of the tumour patient and controls was subjected to RT-PCR and the coding sequence of the DPD cDNA was fully amplified in four fragments that span 737 bp (fragment A), 1010 bp (fragment B), 904 bp (fragment C) and 916 bp (fragment D) (Figure 3, upper panel). Analysis of the PCR fragments by gel electrophoresis showed normal sized fragments A, B and D in all

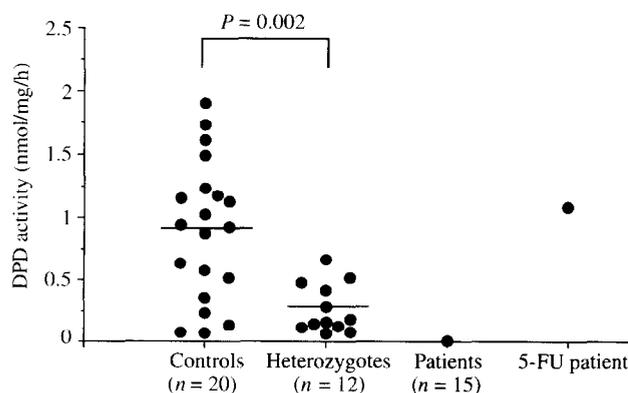


Figure 1. DPD activity in fibroblasts. The mean activities of DPD in controls and obligate heterozygotes are indicated by solid lines.

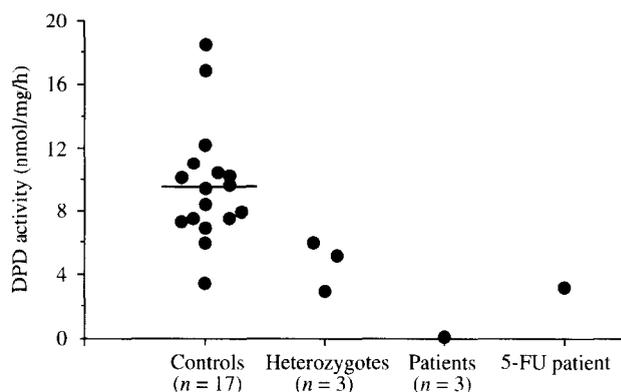


Figure 2. DPD activity in leucocytes. The mean activity of DPD in controls is indicated by a solid line.

subjects (results not shown). However, the 904 bp fragment C was found together with a smaller sized fragment of 739 bp in the tumour patient, whereas only the normal sized 904 bp fragment was detected in a control subject (Figure 3, lower panel). For comparison, we also included the analysis of the 904 bp fragment in a Finnish paediatric patient suffering from a DPD deficiency (subject 2) who proved to be homozygous for the smaller sized fragment, whereas his father (subject 1) proved to be heterozygous for the predicted normal-sized fragment and the smaller-sized fragment.

Sequence analysis of the PCR fragments showed that the 739 bp fragment originated from the 904 bp fragment by a deletion of 165 bp which proved to be identical to the deletion described previously in two unrelated Dutch patients [4–6].

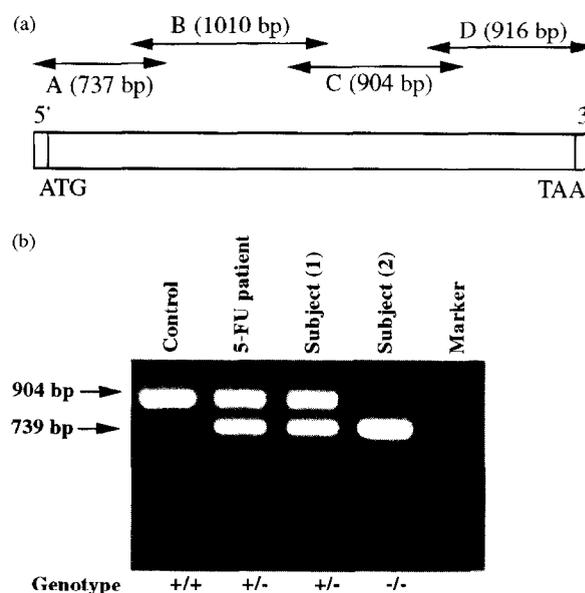


Figure 3. Amplification of DPD mRNA by RT-PCR and analysis by gel electrophoresis. The sequence of the cDNA coding for DPD was fully amplified in four fragments (a). (b) shows the analysis of the C fragment from a control, the 5-FU patient, a paediatric DPD patient (subject 2) and his father (subject 1). The 904 bp and 739 bp bands correspond to the wild-type and the deleted cDNA fragments, respectively. The sizes of the three bands present in the marker lane are 947 bp, 831 bp and 564 bp, respectively. A plus indicates the presence of the wild-type allele and a minus indicates the presence of the mutant allele.

### Genomic sequence analysis

In order to identify the genomic mutation leading to the observed exon skipping in our patients, two intron specific primers were used for amplification of the complete exon and the upstream and downstream intron sequences [6]. Sequence analysis showed that the tumour patient proved to be heterozygous for a G→A point mutation in the invariant GT splice donor sequence in the intron downstream of the skipped exon (Figure 4(b)), whereas a normal conserved GT splice donor site was observed in the control (Figure 4(d)). The paediatric patient suffering from a DPD deficiency proved to be homozygous for the G→A mutation thereby changing the invariant splice donor site GT into AT (Figure 4(a)), whereas his father proved to be heterozygous for this type of mutation (Figure 4(c)).

### DISCUSSION

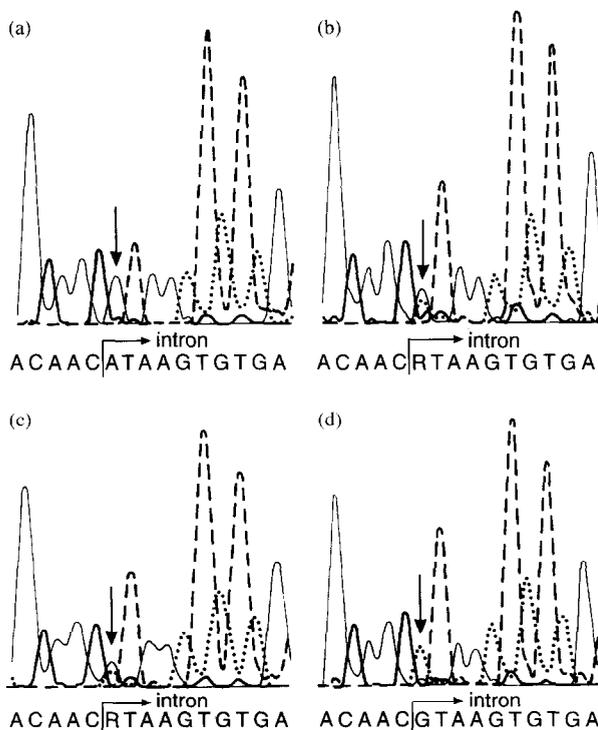
We have provided unambiguous evidence at the molecular level for heterozygosity of a mutated allele of the gene encoding DPD in a tumour patient suffering severe toxicity after administration of 5-FU. Although haematological (leucopenia) and gastrointestinal toxicities (nausea, vomiting, diarrhoea) are common side-effects of 5-FU, the occurrence of hyperpigmentation and cardiotoxicity are rarer side-effects [30–32]. Hyperpigmentation and cardiotoxicity are more often observed after protracted infusion of 5-FU when com-

pared to bolus injections, as a result of the extended infusion of this drug [30–32]. Thus, the occurrence of these types of toxicities in our patient might be related to increased 5-FU levels due to a decreased activity of DPD. Furthermore, our patient might be especially prone to the development of 5-FU-associated cardiotoxicity since she is familiar with coronary artery disease [32].

Surprisingly, determination of the activity of DPD in fibroblasts of the 5-FU patient showed normal activity of DPD, which was within the range of controls and outside the range of DPD activities as observed for obligate heterozygotes. Although fibroblasts are a very suitable type of tissue to establish a complete deficiency of DPD, the overlapping range in DPD activities between controls and obligate heterozygotes precludes unambiguous detection of heterozygotes. It is now well established that the activity of DPD is generally lower in proliferating (malignant) cells than in resting (differentiated) cells [33–35]. Therefore, a conceivable possibility for the large range of DPD activities in fibroblasts might be that the activity of DPD is influenced by the degree of confluency of these fibroblasts. A similar phenomenon has been observed for lysosomal enzyme activities which proved to vary during the various growth phases of human fibroblasts [36]. In that respect, we and others have shown that carrier detection of a deficiency of DPD can be performed by determination of the DPD activity in peripheral mononuclear cells [13, 24, 37]. Analysis of the activity of DPD in the leucocytes of the cancer patient demonstrated that the activity of DPD was comparable to that observed in other obligate heterozygotes. Furthermore, the residual activity of DPD in the leucocytes of the cancer patient (32% of the mean DPD activity of leucocytes obtained from healthy donors) is comparable to that observed in other patients suffering from 5-FU toxicities [17].

Although the activity of DPD can be detected in a variety of human tissues, it should be borne in mind that the majority of the DPD activity, and therefore the catabolism of 5-FU, is confined to the liver [38, 39]. Lu and associates showed in 2 cancer patients suffering from a partial DPD deficiency that the decreased activity of DPD in peripheral mononuclear cells was paralleled by a decreased activity of DPD in the liver as well [17]. Therefore, they suggested that the activity of DPD in peripheral mononuclear cells could be used as a marker for DPD activity in general. However, Stéphan and associates reported a patient with a disease-related liver impairment who suffered from severe toxicities after administration of 5-FU due to a decreased activity of DPD in liver, whereas a normal activity of DPD in lymphocytes was observed [18].

The G→A point mutation changes an invariant GT splice donor site into AT which, apparently, leads to skipping of a 165 bp exon immediately upstream of the mutated splice donor site during the splicing of DPD pre-mRNA. As a consequence, a 165 bp fragment encoding the amino acid residues 581–635 of the primary sequence of the DPD protein is lacking in the mature DPD mRNA [4–6]. So far, the analysis of 11 patients with a complete deficiency of DPD revealed homozygosity for the G→A point mutation in 6 patients from Denmark, Finland and The Netherlands and heterozygosity for this mutation in 2 patients ([5, 6]; data not shown). The apparent high frequency of this mutation among patients with a complete deficiency of DPD supports the observation that there is some kind of homogeneity for this mutation in Northern Europe [24].



**Figure 4. Sequence analysis of the intron-exon boundary.** A genomic DNA fragment spanning the DPD exon and its flanking sequences was amplified and used for direct sequence analysis. (a) Paediatric DPD patient (subject 2); (b) 5-FU patient; (c) father of the paediatric patient (subject 1); (d) control. The sequence depicted below each panel shows the last five nucleotides of the exon (ACAAC) followed by the first 10 nucleotides of the intron. In the paediatric DPD patient, homozygosity for a G→A point mutation in the first nucleotide of the downstream intron (indicated by an arrow) is shown. The father of the paediatric patient and the 5-FU patient are heterozygotes for this mutation, since both G and A are present in this position.

Although analysis of the DPD activity in 124 healthy subjects [17] and 185 cancer patients [23] did not reveal patients with a complete deficiency of DPD, a number of patients have been found with low activities of DPD experiencing increased toxicities, including death, following 5-FU treatment [17]. In this respect, the frequency of patients who are heterozygous for a mutated DPD allele has been estimated to be as high as 3% [20–24]. Considering the frequent use of 5-FU in the treatment of cancer patients, the severe 5-FU-related toxicities in patients with a low activity of DPD and the high frequency of the G→A mutation in DPD deficient patients, analysis of DPD activity in leucocytes and screening for the G→A mutation should be routinely carried out prior to the start of the treatment with 5-FU. Fortunately, the G→A point mutation destroys a unique *MaeII* restriction site present in an amplified genomic DNA fragment encompassing the skipped exon and its flanking sequences, allowing the rapid screening of this mutation in patients [6]. The prevalence of this mutation in cancer patients is currently under investigation in our laboratory.

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