Prevalence of a Common Point Mutation in the *Dihydropyrimidine Dehydrogenase* (*DPD*) Gene within the 5′-Splice Donor Site of Intron 14 in Patients with Severe 5-Fluorouracil (5-FU)-related Toxicity Compared with Controls[1]

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ABSTRACT

Deficiency of dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme in 5-fluorouracil (5-FU) catabolism, has been linked to toxic side effects of 5-FU. The most prominent mutation of the *DPD* gene resulting in severe DPD deficiency is a G to A mutation in the GT 5′-splice recognition site of intron 14 (exon 14-skipping mutation). The corresponding mRNA lacks exon 14, and the enzymatic activity of the translated DPD protein is virtually absent. We developed a reverse transcription-PCR-based assay suitable for routine identification of the exon 14-skipping mutation and screened a control cohort of 851 Caucasian individuals as well as a cohort of 25 cancer patients reported by their physicians to have suffered from WHO grades 3–4 toxicity upon 5-FU chemotherapy. Within the control cohort, in total, eight heterozygotes were detected (0.94%): one heterozygote in 51 healthy donors, (1.96%); five heterozygotes in 572 hospital patients (0.87%); and two heterozygotes in 228 colorectal tumor patients (0.88%). Among the 25 patients with severe 5-FU-related toxicity, 5 (20%) were heterozygous and 1 (4%) was homozygous for the exon 14-skipping mutation. All six patients had experienced WHO grade 4 myelosuppression. Lethal outcome was seen in the homozygous and two of the heterozygous cases. We conclude that carriers of the *DPD* exon 14-skipping mutation are at significantly increased risk to experience life-threatening myelosuppression upon 5-FU treatment, even when the allelic status is heterozygous. These data lead us to suggest routine testing for the exon 14-skipping mutation before 5-FU treatment.

INTRODUCTION

5-FU,[3] a commonly used chemotherapeutic agent, is a pyrimidine analogue, which is metabolized by the pyrimidine metabolic pathways (1). It exerts cytotoxic effects on tumor cells via its metabolites. The 5-FU metabolite 5–5-fluoro-dUMP inhibits thymidylate synthase thus impairing DNA synthesis. Additional cytotoxic mechanisms result from 5-FU metabolites being incorporated into RNA and DNA (1, 2). The amount of 5-FU available for anabolic reactions and subsequent cytotoxicity is determined by its catabolism. Less than 10% of administered 5-FU undergoes renal excretion, whereas >80% is cleared by catabolic degradation (for a recent review, see Ref. 3). The apparent plasma half-life of 5-FU depends strongly on the mode of administration and is characterized by a high intra- and interpatient variability and nonlinear elimination kinetics (4). Nevertheless, 5-FU clearance from plasma proceeds rapidly. After i.v. bolus administration of 500 mg/m^2, for example, 5-FU plasma levels decline with an apparent elimination half-life of ~12 min (5). The rate-limiting initial step of 5-FU catabolism is catalyzed by DPD (for a recent review, see Ref. 3), and deficiency in DPD enzyme activity is correlated with a delay in the clearance of 5-FU from the plasma (6, 7). Accordingly, toxic side effects of 5-FU have been linked to low levels of DPD enzyme activity in human blood cells (6, 8–13).

Human DPD cDNA has been cloned and sequenced, and the genomic structure of the *DPD* gene has been reported (14–16). Subsequently, a number of mutations and polymorphisms have been identified, some of which result in a decreased activity of the DPD enzyme (13, 17–20). The most frequent of these mutations is a G to A point mutation within the 5′-splicing donor site of intron 14 (exon 14-skipping mutation; DPD splice site mutation; Refs. 13 and 19). Processing of pre-mRNA bearing this mutation results in a loss of exon 14. The resulting protein product is truncated by 55 amino acids; its catalytic activity is virtually absent (17). A first report of a heterozygous carrier of the exon 14-skipping mutation who had experienced severe toxicity after 5-FU treatment was published in 1996 (11). Subsequently, additional cases have been reported (13, 21, 22).

[3] The abbreviations used are: 5-FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; RT-PCR, reverse transcription-PCR; UICC, Union International Contre Cancer; WT, wild type.

On the basis of these findings, it has been suggested to screen cancer patients routinely for the exon 14-skipping mutation before 5-FU treatment (11, 13, 21). However, no statistically sound data on the prevalence of this mutation that warranted such a suggestion have been reported to date.

A very recent report provided first data indicating a high frequency (25%) of heterozygotes for the exon 14-skipping mutation among 37 Caucasian cancer patients who had experienced severe 5-FU-related toxicity (13). In all cases, the mutation was linked to low DPD enzymatic activity in peripheral blood mononuclear cells. However, there have been conflicting data based on a group of 37 (presumably French) patients (20). Within this group none of the 23 individuals with reduced DPD activity carried the exon 14-skipping mutation.

Here we report, for the first time, prevalence data showing that the exon 14-skipping mutation is present in ~1% of the Caucasian population. In addition, we confirm, on the basis of a sample of 25 randomly selected cancer patients who had experienced WHO grade 4 toxicity upon 5-FU treatment, that the frequency of the exon 14-skipping mutation within this group is high (24%).

MATERIALS AND METHODS

Patients with WHO Grade 3 and 4 Toxicity during 5-FU Treatment. In a periodical journal for German oncologists (Arbeitsgemeinschaff Internistische Onkologie newsletters), physicians were asked to report cases of severe 5-FU-related toxicity. Oncologists responding between November 1999 and January 2000 received a query sheet together with a detailed (Common Toxicity Criteria WHO) checklist for the categorization of 5-FU-related toxic effects. The physicians were asked to send a blood or tissue sample together with the query sheet containing detailed information concerning 5-FU-related toxicity and the chemotherapy protocol, age, sex, and tumor site. Between November 1999 and January 2000, we received blood samples from 25 patients, fresh-frozen skin tissue from 1 patient, and paraffin-embedded tissue from 9 patients. Ten cases had to be excluded from the study because patients had suffered toxicity with WHO grades <3 ($n = 5$), inaccurate clinical data in the query sheet ($n = 3$), and observed symptoms not related to 5-FU chemotherapy ($n = 2$).

Control Population of Randomly Selected Hospital Patients. The frequency of the heterozygous DPD exon 14-skipping mutation was evaluated in a cohort of randomly selected hospital patients by analyzing leukocytes ($n = 572$), frozen tissue ($n = 95$), or paraffin-embedded tissue ($n = 133$) from 800 Caucasian individuals (see Table 2). The majority (533) of leukocyte samples were from a cell bank of the Department of Internal Medicine II, Friedrich-Schiller-University, Jena, Germany, and had been routinely collected from patients with various hematooncological diseases upon their entry into the clinics. A smaller number of leukocyte samples (39) was from randomly selected colorectal cancer patients (all tumor stages) before chemotherapy. Frozen primary tumor tissue or paraffin-embedded primary tumor tissue was from patients with colorectal lesions (all tumor stages) collected in a tissue bank of Oncoscreen GmbH, Jena, Germany.

Control Population of Healthy Donors. Leukocyte samples were from 51 healthy German volunteers who were randomly selected from among the staff of the Department of Internal Medicine II, Friedrich-Schiller-University and Oncoscreen.

RNA Preparation from EDTA Blood Samples. EDTA-blood was collected, and the leukocytes were isolated by hypotonic lysis using Erythrocyte Lysis Buffer (Qiagen) and subsequent centrifugation. RNA from leukocytes was isolated using TRIzol (Life Technologies, Inc., GmbH, Karlsruhe, Germany) according to the manufacturer's recommendations. Average RNA yields were 6 μg/ml blood, which were in accordance with the value indicated by the manufacturer (6–7 μg/7 × 10⁶ WBCs).

RNA Preparation from Fresh-frozen Tissue. Approximately 5 mg of fresh-frozen tissue were pulverized, and the RNA was extracted using TRIzol (Life Technologies, Inc., GmbH). On average we obtained 15 mg/preparation, which is within the expected range.

RNA Preparation from Paraffin-embedded Tissue. RNA from paraffin-embedded tissue was prepared as described by Coombs *et al.* (23), with modifications. Briefly, 200 μl of xylene (Roth, Karlsruhe, Germany) was added to each 10-μm section, heated for 10 min to 37°C, centrifuged for 3 min at 12,000 × g, and then xylene was removed by aspiration. The xylene washing was repeated three times. The pellet was washed in 100% ethanol and dried. The pellet was rehydrated in a 300-μl reaction buffer (10 mM TRIS; Life Technologies, Inc., GmbH), 5 mM EDTA (Life Technologies, Inc., GmbH), 0.5% *N*-lauroylsarcosine (pH 8.0; Sigma Chemical Co., Taufkirchen, Germany), and mechanically homogenized. Proteinase K (Sigma Chemical Co.) was added to a final concentration of 65 μg/ml. After incubation at 55°C for 3 h, the reaction mix was stopped by addition of sodium acetate (0.6 M final concentration; pH 4.0) and the RNA was extracted with phenol/chloroform/isoamylalcohol according to Chomczynski and Sacchi (Ref. 24; reagents from Life Technologies, Inc., GmbH). For demodification, RNA was incubated for 60 min at 70°C before reverse transcription reaction according to Masuda *et al.* (25). From a 10-μm section (which roughly corresponds to 1 mg of tissue), on average, we obtained 2.4 μg, which was remarkably close to the RNA yield obtained from fresh-frozen tissue.

RT-PCR Analysis. RNA was precipitated, washed twice in 70% ethanol (Roth), dried, resuspended in 40 μl of distilled water, and reverse transcribed with hexamers (Amersham Pharmacia, Freiburg, Germany) into cDNA using MMLV (Life Technologies, Inc., GmbH) according to the manufacturer's instructions. Two μl of cDNA were used in a total 50-μl PCR reaction mix containing 47 μl of PCR Supermix (Life Technologies, Inc., GmbH) and 1 μl primer mix (12 μM each forward and reverse primer). The PCR was run for 35 cycles. The DPD mRNA sequence with the GenBank accession no. U09178 was used, with exon 14 ranging from nucleotide 1822 to 1986. For amplification of cDNA derived from blood and fresh-frozen tissue, primers were selected for amplification of a sequence ranging from nucleotide 1939 to nucleotide 2043, with forward primer F13 starting from nucleotide 1939 and reverse primer R15 starting from nucleotide 2043 (Fig. 1, *A* and *B*). For amplification of cDNA derived from paraffin-embedded tissue,
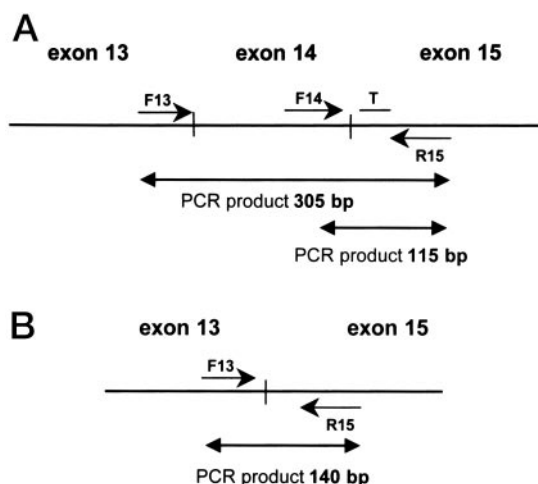
Fig. 1 Primer location for the DPD exon 14-skipping mutation analysis. Using the DPD mRNA sequence (GenBank accession no. U09178), the PCR primers were located on exon 13 (*F13*), exon 14 (*F14*), and exon 15 (*R15*). The PCR products of the mutant allele lacking exon 14 were expected to be shorter (140 bp; *B*) than those of the wild-type allele (305 bp; *A*). A 115-bp amplicon is expected when analyzing paraffin-embedded material using primer set F14/R15 (*A*). A TaqMan probe (*T*) was designed to confirm the homozygous mutant specimen using the primer/probe set F14/T/R15 (*A*).
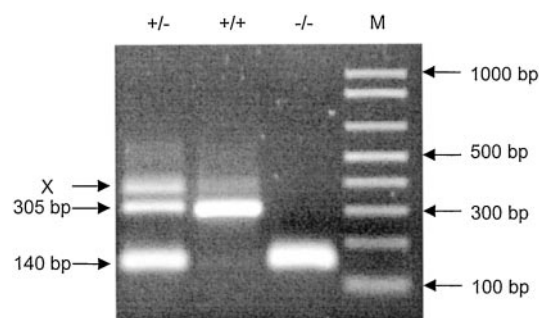

Fig. 2 Gel electrophoresis of PCR products of cDNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) exon 14-skipping mutation allele. *M,* molecular weight standard. The PCR products of wild-type and of the homozygous mutant cDNA result in a band at 305 bp and 140 bp, respectively. The PCR products of the heterozygous background yielded in the expected 305-bp and 140-bp amplicons, but, in addition, in a 390-bp spurious band (×). The band × is a heteroduplex formation of the wild-type and mutant PCR products generated during the PCR in a competitive situation. The cDNA was obtained from fibroblast cell cultures of donors with known genetic backgrounds.

a sequence from nucleotide 1929 to 2043 was amplified with forward primer R14, starting from nucleotide 1929, and reverse primer R15, described above (Fig. 1*A*).

Quantitative real-time TaqMan RT-PCR (Applied Biosystems, Weiterstadt, Germany) of a DPD sequence common to wild-type and mutant molecules was performed as described previously (26). The mutant-specific amplicon was amplified under the same PCR conditions as the common sequence. For RT-PCR of the mutant sequence, the forward primer F14, the reverse primer R15, and a TaqMan Probe located in between, starting from position 1951, were used, thus amplifying a sequence ranging from nucleotide 1929 to nucleotide 2043.

RT-PCR Analysis of cDNA Obtained from Paraffin-embedded Sections. In addition to cDNA from leukocytes, we analyzed cDNA prepared from archival paraffin-embedded tissue to facilitate the analysis of patients who had died during their 5-FU chemotherapy. Preliminary experiments revealed that RNA extracted from paraffin-embedded tissue contained predominantly fragments smaller than 300 bp (data not shown). Although the mutant fragment of 140 bp was easily detectable in these samples, the 305-bp PCR product of the wild-type allele was unlikely to be amplified. For this reason we designed a second PCR reaction with the forward primer located on exon 14 and the reverse primer located on exon 15. PCR resulted in a band of predicted size (115 bp) when cDNA known to contain the wild-type allele was used as a template. RT-PCR-based screening of archival paraffin-embedded tissue thus consisted of two separate RT-PCRs.

RESULTS

RT-PCR Analysis. PCR products obtained from cDNAs of fibroblasts from three donors with different genetic back-

grounds are shown in Fig. 2. As expected (see Fig. 1), PCR products of the mutant allele, which resulted from the skipping of exon 14, were 165 bp shorter than those of the wild-type allele. Amplification of the wild-type allele from a homozygous control person resulted in a PCR product of 305 bp, whereas amplification of the homozygous mutant allele resulted in a band of 140 bp. PCR of cDNA from a person heterozygous for the exon 14-skipping mutation yielded three bands: a band at 140 bp (the PCR product of the mutant allele), a band at 305 bp (the PCR product of the wild-type allele), and a weaker band of 390 bp. The 390-bp band is caused by a heteroduplex formation of the mutant and wild-type amplicon during the PCR, which is a common phenomenon in competitive PCR (27). Only when a high amount of PCR product has accumulated is a very faint band of similar size visible in the wt/wt sample (Fig. 3). However, this band is clearly an artifact of PCR at high template/ product concentration. In contrast with the other bands, it is no longer visible when PCR is limited to 30 cycles. The 390-bp band does not influence the exact analysis of the genotype. A typical result of the RT-PCR-based screening of cDNA obtained from leukocytes is shown in Fig. 3. cDNA of leukocytes from a healthy donor known to be heterozygous for the exon 14-skipping mutation was used as a positive control. The identity of both the 140-bp band and the 305-bp band was confirmed by sequencing.

To screen archival paraffin-embedded tissue, a modified approach was necessary. Gel analysis of RNA derived from paraffin-embedded tissue revealed that this RNA primarily consisted of molecules smaller than 200 bp (data not shown). Because of this degradation, the wild-type amplicon of 305 bp was not expected to be amplified, even in wild-type cDNA with primer set F13/R15. Indeed, PCR results presented in Fig. 4*A* show that the wild-type 305-bp band is detected only in the heterozygous control derived from leukocytes (*Lane 6*) but not in any of the four cDNA specimens derived from paraffin-embedded tissue (Fig. 4*A, Lanes 1–4*). Primer set F13/R15 did, however, give rise to the 140-bp mutant band, even in paraffin-
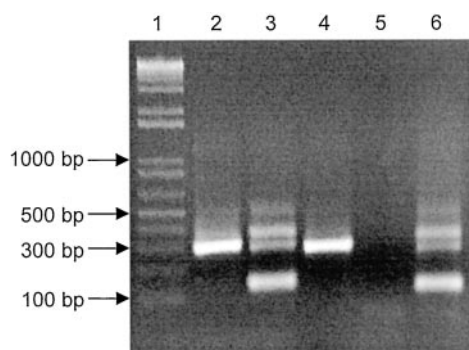
Fig. 3 Example of a screening of cDNA obtained from tumor patients. *Lane 1,* molecular weight standard; *Lanes 2–4,* samples from tumor patients; *Lane 5,* negative control; *Lane 6,* positive control for heterozygous exon 14-skipping (cDNA from fibroblast cell cultures of donors with known genetic background). In *Lanes 2* and *4,* only the 305-bp band is seen; those patients are homozygous wild-type. *Lane 3,* the band at 305 bp is indicative for the wild-type allele, whereas the additional band at 140 bp indicates the exon 14-skipping mutation. Thus, the patient is heterozygous for the exon 14-skipping mutation.

Fig. 4 PCR products of cDNA obtained from paraffin-embedded tissue. *A,* PCR with the same primers used for the experiment shown in Fig. 3. *Lanes 1–4,* cDNA obtained from paraffin-embedded tissue. Because of poor RNA quality, the 305-bp band (indicative of the wild-type allele) was not amplified. *Lane 4,* the 140-bp band of the mutant allele was amplified. All *lanes* except *Lane 6* (positive control) show primer dimer (*P*) below 100 bp. *B,* an additional PCR specific for the wild-type allele with a predicted product of 115 bp. Note the presence of the 115-bp band in all *lanes* except the negative control (*Lane 5*). Thus the patient of *Lane 4* is heterozygous for the exon 14-skipping mutation. *Lane 7,* molecular weight standard; *Lanes 1–4,* unknown samples from tumor patients; *Lane 5,* negative control; *Lane 6,* positive control for heterozygous exon 14-skipping (not from paraffin-embedded tissue).

derived cDNA samples (Fig. 4*A, Lane 4*). Thus, an additional primer set F14/R15 (see Fig. 1*A*) was designed to amplify a 115-bp band specific for wild-type molecules only. Fig. 4*B* shows that this 115-bp wild-type sequence was amplified in all four cDNAs derived from paraffin-embedded tissue as well as in the positive control cDNA. Thus, the specimen in *Lane 4* was heterozygous, and the other three paraffin-derived specimens were homozygous for wild-type.

Quantitative TaqMan RT-PCR Analysis of a Homozygous Mutant Specimen. One paraffin-embedded specimen (Table 1, patient 8) was proved to be a homozygous mutant using the approach described above. Quantitative TaqMan RT-PCR on the ABI 7700 Prism machine was applied to confirm this result. The TaqMan signal derived from a common sequence (26) was compared with the TaqMan signal derived from the wild-type-specific PCR product obtained with the primer set F14/R15 (Fig. 1*A*). A TaqMan RT-PCR signal was detected around cycle 27 for the common DPD sequence of all specimens. There was a sound wild-type-specific TaqMan RT-PCR signal around cycle 30 from paraffin-derived cDNA previously tested to be wt/wt (Table 1, patient 13) or heterozygous (Table 1, patient 19). However, no wild-type-specific TaqMan signal was detectable from patient 8 after 40 PCR cycles.

Frequency of DPD Exon 14-skipping Mutation among Patients with 5-FU Toxicity. RT-PCR analysis of mRNA samples from 25 patients reported by their physicians to have suffered from WHO grade 3 or 4 toxicity related to 5-FU chemotherapy revealed 1 patient (4%; 95% confidence interval: 2.0–6.0%) to be homozygous and 5 patients (20%; 95% confidence interval: 8.0–32.0%) to be heterozygous for the DPD exon 14-skipping mutation (Table 1). The remaining 19 patients (76%) did not carry this mutation.

Clinical Presentation of the Patient Homozygous for the DPD Exon 14-skipping Mutation. Patient 8, a woman 48 years of age with postmenopausal breast cancer (American Joint Committee on Cancer stage IIA), was treated with cyclophos-
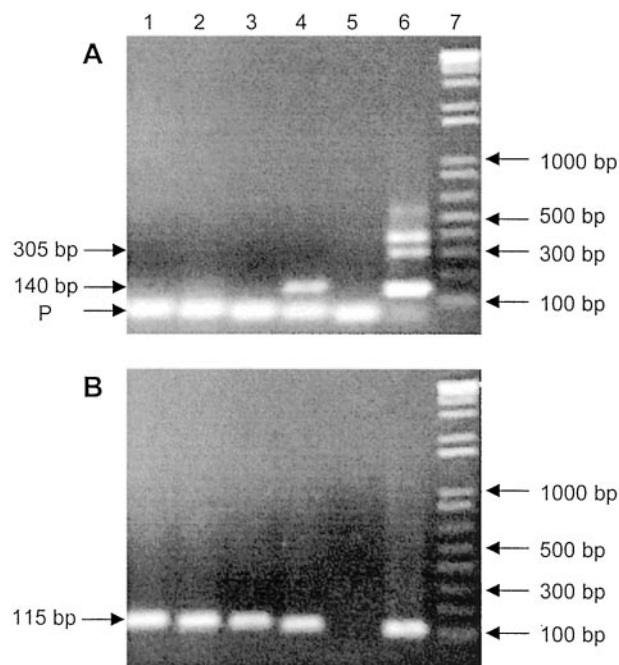
phamide (400 mg/m^2), methotrexate (40 mg/m^2), and 5-fluorouracil (600 mg/m^2) on days 1 and 8 in an adjuvant setting. Three days after completion of the first cycle, she experienced acute bone marrow aplasia. Broad-spectrum antibiotics and granulocyte colony-stimulating factor were administered because of febrile neutropenia. Despite these treatments at the intensive care unit, she suffered from a rapid, progressive, therapy-resistant pneumonia and developed cardiopulmonary failure. The patient died in a multiorgan failure 12 days after the end of the first chemotherapy cycle.

Clinical Presentation of Patients Heterozygous for the DPD Exon 14-skipping Mutation. Patient 14, a man 63 years of age with UICC stage III colon cancer, received chemotherapy with 5-FU (425 mg/m^2) and folinic acid (20 mg/m^2) for 5 days as an adjuvant treatment. Ten days after completion of the first cycle, the patient was readmitted to the hospital with leukopenia WHO grade IV, sepsis, and disseminated intravascular coagulation. The patient died 4 days after admission from multiorgan failure.

Patient 19, a man 78 years of age with pulmonary metastasis of a colon cancer, had palliative treatment with 5-FU (425 mg/m^2) and folinic acid (20 mg/m^2) for 5 days. Five days after

Table 1 Characteristics and DPD exon 14-skipping allelotype of 25 cancer patients with severe 5-FU related toxicity

Patient	Sex	Age	Tumor	Chemotherapy	Lethal outcome of 5-FU related toxicity	Main toxicity	Exon 14 skipping
1	w	66 yr	Rectal cancer	5-FU radiochemotherapy	No	Leucopenia (4), diarrhea (4)	No
2	m	58 yr	Colon cancer	5-FU/FA	No	Stomatitis (3)	No
3	m	68 yr	Colon cancer	5-FU/FA	No	Stomatitis (3), diarrhea (3)	No
4	w	57 yr	Gastric cancer	5-FU continuous infusion	No	Stomatitis (3)	No
5	w	63 yr	Colon cancer	5-FU/FA	No	Stomatitis (4), diarrhea (4), alopecia (3)	No
6	m	58 yr	Gastric cancer	5-FU/cisplatin	No	Emesis (3), Stomatitis (4)	No
7	m	73 yr	Rectal cancer	5-FU	No	Leucopenia (4)	Heterozygous
8	w	48 yr	Breast cancer	Cyclophosphamide, methotrexate, 5-FU	Yes	Leucopenia (4)	Homozygous
9	m	57 yr	Colon cancer	5-FU/FA	No	Leucopenia (4)	No
10	w	70 yr	Colon cancer	5-FU/FA	Yes	Leucopenia (4)	No
11	m	74 yr	Rectal cancer	5-FUt	No	Leucopenia (4)	No
12	w	48 yr	Colon cancer	5-FU/FA	No	Leucopenia (4), mucositis (3)	No
13	w	72 yr	Colon cancer	5-FU/FA	Yes	Leucopenia (4)	No
14	m	63 yr	Colon cancer	5-FU/FA	Yes	Leucopenia (4), diarrhea (3)	Heterozygous
15	m	71 yr	Colon cancer	5-FU/FA	Yes	Leucopenia (4), diarrhea (4)	No
16	w	68 yr	Breast cancer	Cyclophosphamide, methotrexate, 5-FU	Yes	Leucopenia (4)	No
17	w	67 yr	Rectal cancer	5-FU chemoradiotherapy	No	Leucopenia (4)	No
18	w	50 yr	Rectal cancer	5-FU	No	Leucopenia (4), mucositis (3)	Heterozygous
19	m	78 yr	Colon cancer	5-FU/FA	Yes	Leucopenia (4), thrombopenia (4)	Heterozygous
20	w	64 yr	Rectal cancer	5-FU/FA	No	Leucopenia (3), diarrhea (4), stomatitis (3)	No
21	m	70 yr	Colon cancer	5-FU/FA	No	Leucopenia (3), stomatitis (4)	No
22	m	43 yr	Rectal cancer	5-FU/FA high dose	Yes	Leucopenia (4)	No
23	w	65 yr	Rectal cancer	5-FU/FA	No	Leucopenia (4)	No
24	w	72 yr	Colon cancer	5-FU/FA	No	Leucopenia (4), mucositis (4)	Heterozygous
25	w	74 yr	Colon cancer	5-FU/FA	No	Leucopenia (4), thrombopenia (4), stomatitis (4)	No

the end of the first chemotherapy cycle, the patient was readmitted to the hospital with a photodermatosis. During the subsequent week, the patient developed severe neutropenia and thrombopenia. Despite intensive care treatment, he died from *Staphylococcus aureus* sepsis in a myocardial failure 8 days after admission.

Patient 7, a man 73 years of age with UICC stage III rectal cancer, was treated with 5-FU (500 mg/m^2) for 5 days in an adjuvant setting. After completion of the first cycle, the patient developed WHO stage IV neutropenia and sepsis. After treatment with broad-spectrum antibiotics and cytokines the patient recovered.

Patient 18, a woman 50 years of age with a UICC stage III rectal cancer, was treated with adjuvant 5-FU (500 mg/m^2) for 5 days. Therapy had to be terminated after the first cycle because of WHO grade IV mucositis and neutropenia. In-patient treatment was needed for 2 weeks, with administration of i.v. antibiotics and cytokines until the patient recovered.

Patient 24, a woman 72 years of age with a double carcinoma of the sigma (UICC stage III) and coecum (UICC stage III), was treated with adjuvant 5-FU (425 mg/m^2) and folinic acid (20 mg/m^2) for 5 days. Therapy had to be terminated after the first cycle because of WHO grade IV mucositis and neutropenia. After a period of 8 days, the patient gradually improved, returning to her previous state of health.

Frequency of the DPD Exon 14-skipping Mutation in the Caucasian Control Population. In an attempt to obtain reliable information on the frequency of the exon 14-skipping mutation within the Caucasian population, cDNA from a total of 851 individuals was tested for the presence of the mutation. Eight of 851 (0.94%) specimens were heterozygous for the exon 14-skipping mutation (Figs. 3 and 4; see Table 2 for details). The remaining 843 individuals (99.06%) were homozygous for the wild-type allele. None of the individuals tested was homozygous for the DPD exon 14-skipping mutation.

One of two heterozygote mutants initially detected on the basis of paraffin-embedded tumor tissue was confirmed by a blood specimen. From the second patient, no blood could be obtained.

To rule out that heterozygote PCR signals from malignant blood specimens were caused by cells of a homozygote wild-type contaminated by somatically mutated tumor cells, a titration experiment was performed. cDNA of a known heterozygote was added to wild-type cDNA in various proportions. A PCR was performed from this titration series and compared with PCR products of five heterozygotes detected within the cohort of 572 blood specimens. The results presented in Fig. 5 indicate that the ratio wild-type:mutant sequence clearly is 1:1 in all five patients; thus, they are real heterozygotes.

One heterozygote individual detected within this cohort of 851 Caucasians was healthy, and seven individuals had malignant disorders: colon cancer (3), breast cancer (1), myeloproliferative syndrome (1), non-Hodgkin's lymphoma (1), and Hodgkin's disease (1). Two of the patients were detected retrospectively to have been treated with 5-FU (infusion) after sampling. Both developed 5-FU-related WHO grade 3 diarrhea.

Table 2 Subcohorts of randomly selected Caucasian individuals screened for the exon 14-skipping mutation

| Source of cDNA	Total no. of specimens	No. of heterozygous individuals	Frequency of the mutation	Confidence interval (95%)
Leucocytes/healthy individuals	51	1	1.96%	1.0%–2.9%
Leucocytes/colorectal patients	39	1	2.56%	1.3%–3.8%
Leucocytes/various patients	533	4	0.75%	0.2%–1.3%
Frozen colorectal tumor	95	0	0.00%	0.0%–0.0%
Paraffin conserved colorectal tumor	133	2	1.50%	0.2%–3.0%
Total	851	8	0.94%	0.4%–1.5%
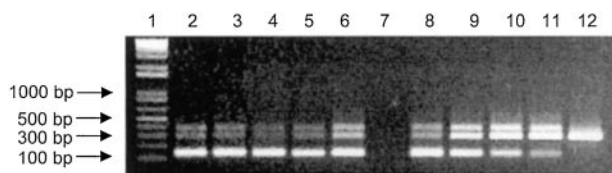
Fig. 5 Titration of mutant and wild-type sequences and of heterozygote specimens. PCR with the same primer set used for the experiment shown in Fig. 3. *Lane 1,* molecular weight standard. *Lanes 2–6,* 5 cDNA preparations of the cohort of 572 blood specimens. *Lane 7,* negative control. *Lanes 8–12,* cDNA of a known heterozygous individual was mixed with the cDNA of a wild-type individual at different proportions (mass of total RNA as determined by the A260 method): only heterozygote (*Lane 8*); heterozygote:wildtype, 1:2 (*Lane 9*); heterozygote:wildtype, 1:4 (*Lane 10*); heterozygote:wildtype, 1:8 (*Lane 11*); only wild-type (*Lane 12*).

DISCUSSION

A point mutation (G to A) within the GT 5'-splicing consensus sequence of exon 14 of the human *DPD* gene has been shown to result in the skipping of the entire exon 14. This exon-skipping causes a deletion of 55 amino acid residues in the primary sequence of the DPD protein resulting in a lack of functional DPD expression (11, 15, 17, 28). The exon 14-skipping mutation is the most abundant loss-of-function allele of the *DPD* gene known thus far (13, 19). Several reports have been made on the prevalence of the exon 14-skipping mutation in different ethnic backgrounds. However, the sample size in all of these reports was too small to yield reliable and consistent data, thus leading to conflicting conclusions. Carriers of the mutant allele were reported in healthy individuals of the following cohorts studied: Finnish (2 of 45 heterozygotes); Taiwanese (2 of 36 heterozygotes; Ref. 11); and Finnish (2 of 90 heterozygotes; Ref. 16). In addition, one heterozygote was detected in a sample of 72 colorectal cancer patients (18). In other small cohorts of healthy donors, however, no carrier of the mutant allele was detected: British (30 individuals; Ref. 11); Taiwanese (131 individuals); Japanese (90 individuals); African-American (105 individuals; Ref. 16); British (30 with low and 30 with high DPD catalytic activity in blood mononuclear cells; Ref. 29); and Dutch (50 individuals; Ref. 28).

We now present data indicating that the mutation occurs with a frequency of 0.94% within the Caucasian population. These findings are based on 851 donors, the largest cohort screened thus far. In the light of our data, it is obvious why the results of previous studies based on sample numbers between 30 and 131 have been conflicting. We detected the mutation in a

heterozygous allelic state in 1 of 51 leukocyte specimens from healthy donors (1.96%), in 5 of 572 leukocyte specimens from patients with various hematooncological disorders (0.87%), and 2 of 228 colorectal tumor specimens (0.88%). No homozygous mutant was detected in our screening study. One may suspect a bias among the donors with malignant disorders, because somatic exon 14-skipping mutations might arise within the tumor tissue, thus resulting in false-positives. However, we were able to rule out this possibility. Thus, the value of nearly 1% heterozygote mutant individuals is representative of the prevalence within the Caucasian population.

To test the hypothesis of heterozygote patients being at significantly increased risk of severe 5-FU-related toxicity, we screened for the occurrence of the exon 14-skipping mutation a cohort of cancer patients who had experienced WHO grade 3–4 toxicity upon 5-FU treatment. The specimens were recruited randomly from German physicians who had reported cases of 5-FU-related WHO grade 3–4 toxicity. Five of 25 patients were heterozygous and one was homozygous for the exon 14-skipping mutation. The homozygote and two of the heterozygotes experienced therapy-resistant sepsis with lethal outcome. WHO grade 4 leukopenia was observed in all of the patients carrying the DPD exon 14-skipping mutation, independent of their allelic status.

A previous report based on a cohort of 37 cancer patients has posed doubts about a high prevalence of the exon 14-skipping mutation among DPD-deficient patients (20). Our data, however, correspond to a second study published very recently (13), which also presents evidence that 25% of patients with WHO grade 3–4 deficiency are heterozygous for the exon-skipping mutation. These data are remarkably close to the results we present independently here.

We have to emphasize, however, that the exon 14-skipping mutation clearly is not the only cause for severe or lethal toxic 5-FU side effects (Table 1). These findings are in line with recent reports: 43% of 5-FU-related toxicity probably are not attributable to DPD deficiency (13), and, in addition to the exon 14-skipping, other loss-of-function mutations of the *DPD* gene have been detected (13, 20, 30, 31). However, exon 14-skipping accounts for approximately one-half of DPD deficiency, rendering this mutation attractive for routine screening (13, 19). Our report adds additional evidence that the exon 14-skipping mutation is very common among patients who suffer from WHO grade 4 leukopenia.

We conclude that approximately one-fourth of patients who experience severe 5-FU-related toxicity carry the exon 14-skipping mutation, with the majority of them being heterozy-

gous. The data presented here and by others (13) strongly indicate that these patients are at high risk of experiencing severe to life-threatening toxicity upon 5-FU treatment. On the basis of a prevalence of nearly 1% within the Caucasian population, it therefore appears that routine genetic screening for this common splice-site mutation should be carried out before 5-FU chemotherapy.

However, it should be pointed out that not everybody who is heterozygous for the mutation may actually suffer severe 5-FU side effects. The individual risk of heterozygous patients suffering from severe or life-threatening 5-FU-related toxicity can only be addressed by pharmacokinetic measurements of the individual 5-FU turnover in heterozygous patients using very low doses of 5-FU. Thus, routine screening for the exon 14-skipping mutation and subsequent individual determination of the 5-FU pharmacokinetics of heterozygous patients provides a concept of individualized therapy and allows the avoidance of undesired treatment toxicity.

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