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Absence of large intragenic rearrangements in the *DPYD* gene in a large cohort of colorectal cancer patients treated with 5-FU-based chemotherapy

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Dihydropyrimidine dehydrogenase (DPD) is the enzyme responsible for the elimination of approximately 80% of the administered dose of 5-fluorouracil (5-FU).
- Mutations in the DPD-coding gene have been shown to increase the risk of severe toxicity in 5-FU treated patients.
- The IVS14+1G>A is the most common DPYD mutation.

WHAT THIS STUDY ADDS

- The intragenic rearrangements of DPYD using multiplex ligation-dependent probe amplification (MLPA) were studied for the first time in a large series of 234 colorectal cancer patients treated with 5-FU-containing chemotherapy.
- No deletions or duplications of one or more DPYD exons were detected. The presence of the IVS14+1G>A mutation was also excluded.
- These data show that neither the large genomic rearrangements in the *DPYD* gene nor the IVS14+1G>A mutation are responsible for the serious toxicity associated with a 5-FU containing regimen in this cohort of Spanish patients.

AIMS

To study the relationship between the toxicity associated with a 5-FU-based therapy and the presence of (i) the large intragenic rearrangements in the *DPYD* gene and (ii) the IVS14+1G>A mutation.

METHODS

We used the multiplex ligation-dependent probe amplification technique (MLPA) to study genomic DNA from 234 colorectal cancer patients treated with 5-FU-based chemotherapy.

RESULTS

We did not detect any deletion/duplication in the *DPYD* gene. The presence of the IVS14+1G>A mutation was also excluded.

CONCLUSIONS

Neither the large genomic rearrangements in the *DPYD* gene nor the IVS14+1G>A mutation play a significant role in the development of serious toxicity associated with a 5-FU containing regimen.

Introduction

For over 50 years, 5-fluorouracil (5-FU) has been the mainstay of chemotherapy for various solid cancers [1]. After intravenous administration of the drug about 80–90% of the dose is catabolized in the liver by dihydropyrimidine dehydrogenase (DPD). The formation of the inactive 5-fluoro-5,6-dihydrouracil (5-FUH2) by DPD is the rate-limiting step of 5-FU catabolism [2]. Although the benefits of 5-FU chemotherapy are well established, the development of severe toxicity is a major clinical problem. A meta-analysis of more than 1000 colorectal cancer patients receiving 5-FU demonstrated that grade 3–4 toxicity exists in 31–34% of patients and is responsible for 0.5% mortality [3]. Several studies have shown that this severe 5-FU related toxicity is clearly associated with DPD deficiency [4–6].

The150 kb *DPYD* gene is located on chromosome 1p22 and comprises 23 exons that range in size from 69 to 1404 pb [7, 8]. The *DPYD* gene is highly polymorphic, and several mutations resulting in a protein with impaired activity have been described [9]. In Western populations, the most frequent of these mutations (IVS14+1G>A) is a G to A point mutation that affects the splice recognition sequence of intron 14 and results in a deletion of 55 amino acids in the native protein [10, 11]. Recently, the precise genomic position of the common fragile site FRA1E was mapped to the chromosomal band 1p22. The authors showed that FRA1E extends over 370 kb within the *DPYD* gene, which genomically spans approximately 840 kb. The 185 kb region of the highest fragility, which accounts for 86% of all observed breaks at FRA1E, encompasses the central part of *DPYD* including exons 13–16 [12]. Considering that fragile sites are prone to breakage, and deletions, translocations and amplifications can therefore occur in these genomic regions, we used the multiplex ligation-dependent probe amplification (MLPA), a simple and reliable method for the detection of intragenic rearrangements [13], to identify the presence of the large intragenic rearrangements in the *DPYD* gene. The MLPA protocol used allowed the simultaneous detection of the IVS14+1G>A mutation.

The study includes a cohort of Spanish cancer patients, 44.4% of whom suffered grade 3–4 adverse effects related to 5-FU administration.

Methods

Patients

Blood samples from 234 colorectal cancer patients undergoing 5-FU-based treatment were collected. All patients gave written informed consent and the study was approved by the Institutional Ethics Committee.

Patients were treated with either (i) 5-FU (a pulse dose of 400 mg m^{-2} on days 1 and 2 and a continuous infusion for 44 h of 1200 mg m^{-2}) with leucovorin i.v and oxaliplatin (85 mg m^{-2} infused for 2 h every 2 weeks i.v.) (FOLFOX) or (ii) 5-FU (2250 mg m^{-2} in 48 h continuous infusion i.v.) with leucovorin i.v and irinotecan (180 mg m^{-2} every 2 weeks i.v.) (FOLFIRI). Pharmacogenetic markers of DNA repairing genes were previously analyzed in the group of patients under FOLFOX treatment [14]. Some of the patients under FOLFIRI treatment were included in a previous study [15]. Patients underwent chemotherapy cycles until severe toxicity developed or disease progression appeared.

Relevant clinical data were obtained from clinical records. Toxicity was graded in accordance with the WHO scale: presence and grade of nausea, asthenia, mucositis, diarrhoea, infection, neutropenia, anaemia and thrombocytopaenia.

MLPA analyses

Genomic DNA was isolated from peripheral leukocytes by the salting-out procedure [16]. DNA (100 ng) was used for MLPA analysis performed with the SALSA MLPA P103 DPYD kit (MRC-Holland) under conditions specified by the manufacturer [17]. SALSA MLPA probes were present for all 23 exons. In view of the large size of the introns, two probes were present for 12 of the exons, and a probe that was specific for the IVS14+1G>A mutation was also present. Amplified PCR products were separated in the 36 cm capillary filled with POP-7 polymer on an ABI PRISM 3130 analyzer (Applied Biosystems). Data were analyzed using Gene Mapper 4.0 software (Applied Biosystems). Coffalyser software developed at MRC-Holland was used to normalize MLPA data. The peak area was normalized by dividing by the sum of areas of all peaks in that lane. Expected normalized values were 1.0 in the absence of copy number change, and 0.5 and 1.5 in the case of heterozygous deletion and duplication, respectively. Figure 1 shows the normal pattern of the *DPYD* gene MLPA analysis and the normalized MLPA results.

EuroGentest, an EU-funded Network of Excellence, has performed an inter-laboratory collaborative validation study of the MLPA method. In the validation report, data on specific performance parameters such as repeatability, reproducibility, accuracy, sensitivity and specificity are shown [18].

To confirm the absence of the IVS14+1G>A mutation in the DNA samples of our patients, we screened them by means of Real-Time PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City. CA.USA). We used the TaqMan® SNP Genotyping Assay (ID: C30633851) to study rs number 3918290 that corresponds to the mutation. Analysis of the amplification reaction was undertaken using the Sequence Detector software, version 2.0 (Applied Biosystems). Data were analyzed using the Allelic Discrimination Program (Applied Biosystems). Ten randomly selected DNA samples were sequenced to confirm the results.

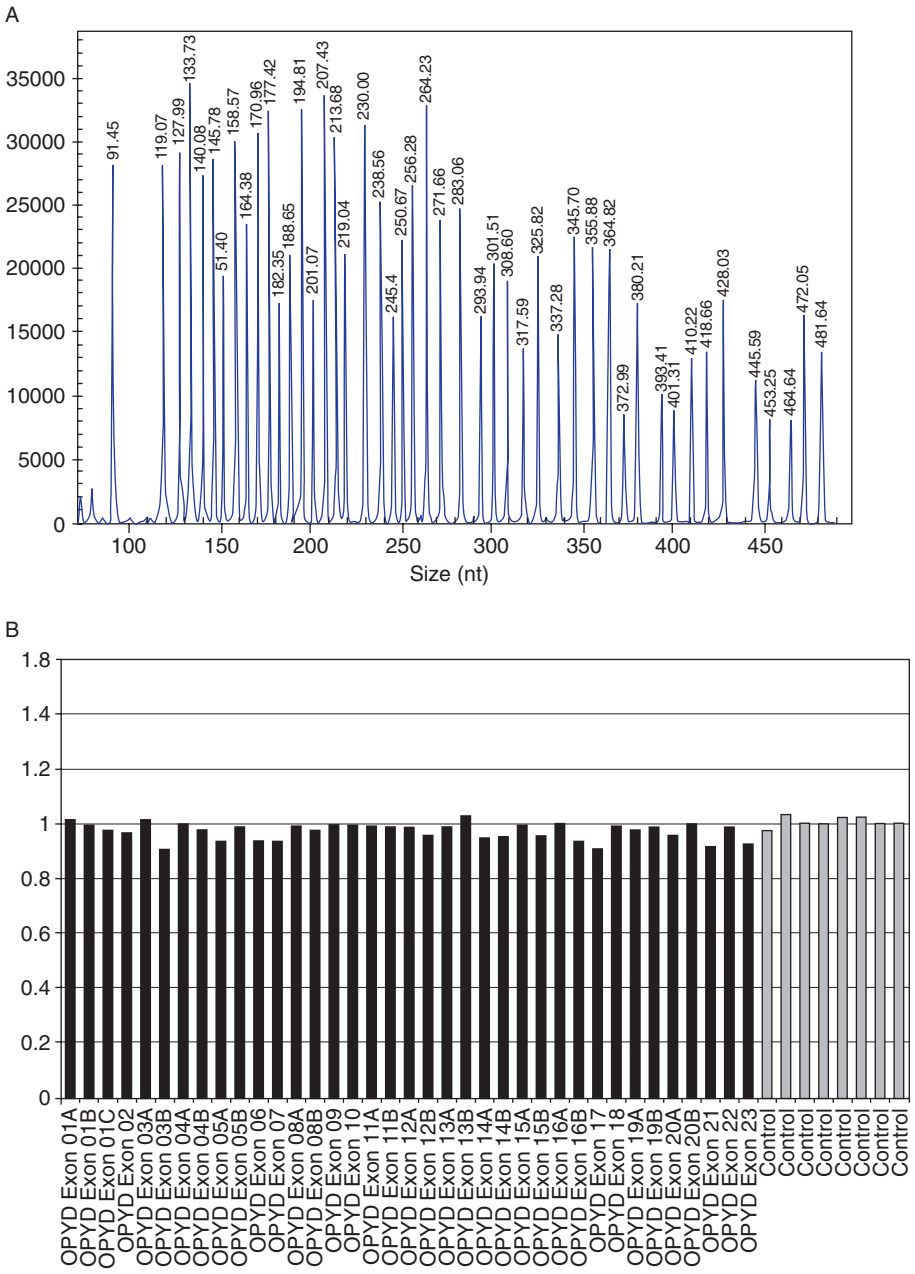

Figure 1

A) Normal pattern of the *DPYD* gene MLPA analysis. There are 40 MLPA probes for the *DPYD* gene (12 exons were covered by two probes and three probes were present for exon 1. The 436 nt probe will only generate a signal on DNA samples containing the IVS14+1 G>A mutation) and eight control probes. On the x-axis all these probes are indicated. B) Normalized MLPA results where the y-axis shows the relative probe peak height as an indication of copy number. Normalized values were 1.0 in the absence of copy number change, and 0.5 and 1.5 in the case of heterozygous deletion and duplication, respectively

Results and discussion

Severe toxicities were frequent in this group of patients. One hundred and four patients (44.4%) suffered a grade 3–4 adverse effect. The most frequent were asthenia (34.6%), nausea (14.5%), diarrhoea (23%) and neutropenia (40.2%) (Table 1).

In our MLPA studies, no differences were found in signal intensities of 15 probes covering the whole FRA1E site (exons 9–18) or in seven probes located in maximum breakage region (exons 13–16). Thus, no deletion/duplication of DPYD exons was found in any of the 234 patient samples.

Few studies have investigated the *DPYD* gene using MLPA [19]. A recent clinical genetic study [1] reported that large intragenic rearrangements of the *DPYD* gene were present in five severely affected patients from a series of 72 patients with complete DPD deficiency. This report dem-

Table 1

Baseline characteristics of the 234 patients

Gender (men/women)	150 (64%) 84 (36%)
Median age (range, years)	64 (24–83)
Performance status	
0–1	182 (77%)
>1	52 (23%)
Therapeutic regimen	
FOLFOX	124 (53%)
FOLFIRI	110 (47%)
Number of cycles of chemotherapy	
Median (range)	9 (1–20)
Cases with toxicity grade 0–2	10 (3–20)
Cases with toxicity grade 3–4	8 (1–20)
Primary tumour localization	
Colon	84 (36%)
Rectum – Sigma	150 (64%)
Toxicity grade 3–4*	
Nausea	34 (14.5%)
Diarrhoea	54 (23%)
Neutropenia	94 (40.2%)
Asthenia	81 (34.6%)

*Patients who developed high toxicity only.

onstrated for the first time the presence of large deletions in the *DPYD* gene in these DPD deficient cases. In a pharmacogenetic context, Ticha *et al*. [20] studied 68 cancer patients treated with a 5-FU containing regimen who had developed high-grade gastrointestinal and/or haematological toxicity. The MLPA analysis of the *DPYD* gene in these patients did not find any deletion/duplication although the IVS14+1G>A mutation was identified in five cases. Similarly, in the present work that includes a large number of colorectal cancer patients, no duplications or deletions were found, indicating that the large genomic rearrangements in the *DPYD* gene do not play a significant role in the toxicity developed by 44.4% of the cases included in the study.

A number of studies have been published concerning the involvement of the IVS14+1G>A mutation in the 5-FU related toxicity. Raida *et al*. studied the prevalence of the splice mutation IVS14+1G>A in 850 Caucasian individuals and in a cohort of 25 cancer patients with grades 3–4 toxicity on 5-FU. The mutation was identified with a frequency of about 1% in Caucasian control individuals. In the group of patients, 20% were heterozygous and 4% were homozygous for the mutation. The authors concluded that carriers of this mutation were at significantly increased risk to experience life-threatening effects on 5-FU treatment, even when the allelic status was heterozygous [10]. Van Kuilenburg *et al*. evaluated the DPD activity and the prevalence of the common splice site mutation IVS14+1G>A in blood samples obtained from 60 cancer patients suffering from severe grade 3–4 toxicity after the administration of 5-FU. A decreased DPD activity was detected in peripheral blood mononuclear cells in 60% of the cases. Furthermore, a high prevalence of the IVS14+1G>A mutation was noted as 28% of all patients were heterozygous or homozygous for this mutation. In patients with a low DPD activity, 42% were heterozygous and one patient (3%) was homozygous for the IVS14+1G>A mutation. In contrast, the IVS14+1G>A mutation was detected in only one out of 24 (4%) patients with a normal DPD activity [11]. To investigate the role of genetic and nongenetic factors for fluorouracil treatment-related severe toxicity, a prospective clinical trial by the German 5-FU Toxicity Study Group was performed. The study included 683 patients with cancer treated with FU monotherapy. Analysis according to toxicity type revealed a significant association of the IVS14+1G>A mutation in the *DPYD* gene with mucositis and leucopenia The mutated allele was found heterozygously in 13 out of 683 patients (1.9%) and there was a clear relationship with gender. Five of the six carriers with severe toxicity were men, whereas six of the seven carriers who did not develop toxicity were women [21]. In a recent paper [22], Magne *et al*. analyzed both DPD activity and the IVS14+1G>A mutation in 131 case-reports with 5-FU-related toxicity. A very low incidence (2%) of the mutation was observed and the authors stated that IVS14+1G>A mutation screening has limited effectiveness in identifying patients at risk for severe 5-FU toxicity in a French population.

In this work, using the MLPA method, no differences were found in signal intensity of the specific probe for the IVS14+1G>A mutation. As the presence of the IVS14+1G>A mutation was excluded, we can conclude that this mutation is not associated with the development of serious toxicity associated with a 5-FU containing regimen in this large group of Spanish patients.

Competing interests

There are no competing interests to declare.

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