Involvement of Target Gene Polymorphisms in 5-Fluorouracil Toxicity: A Case Report

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Abstract
Personalized medicine is becoming an important tool in oncology, both in preventing disease and in optimizing the treatment of existing cancers. Here we describe the cases of 2 patients with relevant systemic toxicity following 5-fluorouracil (5-FU) therapy and we study the more frequent polymorphisms in the target genes, in particular: (1) the variability in the number of 28-base repetitions present in the 5’-untranslated sequence of the thymidine synthase gene; (2) the presence of single-nucleotide polymorphisms in the methylene tetrahydrofolate reductase gene, and (3) the presence of mRNA splicing in intron 14 of the hepatic enzyme dihydropyrimidine dehydrogenase. The 5-FU gene profile of our patients strongly suggested that the polymorphisms expressed may contribute to the adverse effects seen during the therapy. To what extent these polymorphisms induced adverse effects cannot be established at present; however, our results strengthen the relevance of the 5-FU-related pharmacogenomic profile to predict the response outcome and the chemotherapy toxicity.

Introduction
It is widely accepted that the future of therapy in oncology lies in personalized medicine, both in preventing disease and in the treatment of existing cancers [1, 2]. Identification of genes associated with the occurrence and prognosis of a variety of cancers will lead to optimized treatment selection. Accordingly, adverse effects seen in drug therapy may be linked to patient genetic variability, both in the target genes and/or in the metabolic enzymatic profile. By identification of target gene polymorphisms, a personalized therapy could be designed, i.e. dose modification, use of equivalent therapies for those at risk or avoidance of a drug therapy if the individual risk outweighs the benefits [3, 4].

Here we describe the cases of 2 patients with relevant systemic toxicity following 5-fluorouracil (5-FU) therapy. 5-FU is a drug belonging to the fluoropyrimidine family, broadly used as the backbone of many combination chemotherapies [5, 6]; indeed, 5-FU indications include palliative and adjuvant treatment of a number of cancers, including colorectal, breast, head and neck cancers [6]. The mechanism of action of 5-FU requires the enzymatic conversion to the nucleotide floxuridine monophosphate that is incorporated into RNA or DNA and exerts its cytotoxic activity. A second mechanism, which is likely the dom-
nant one, involves thymidylate synthase (TS) inhibition through the active metabolites fluorodeoxyuridine monophosphate (FdUMP) or 5-fluoro-dUMP. This reaction is facilitated by the formation of a ternary complex consisting of 5-fluoro-dUMP, TS and the folate derivative cofactor 5,10-methylene tetrahydrofolate (5,10-MTHF). The 5,10-MTHF and FdUMP form a covalently bound complex with TS [6]. The MTHF intracellular levels are regulated by the enzyme MTHFR (methylene tetrahydrofolate reductase) [7]. Despite its clinical benefit, 5-FU is associated with frequent gastrointestinal and hematologic toxicities, which often leads to treatment discontinuation. Both genetic and nongenetic factors have been associated with 5-FU toxicity, and a number of genes involved in 5-FU pharmacokinetics and pharmacodynamics have been shown to affect 5-FU treatment outcomes [8, 9, 11]. Genome-based studies have shown that approximately 26–65% of human variations in susceptibility to 5-FU-induced cytotoxicity are due to genetic components [10]. Indeed, enzymes involved in the 5-FU mechanism of action as well as those involved in its metabolism show polymorphisms at the genetic level that influence the structure and function of the encoded protein [8, 9, 11]. In addition, both age and sex have been shown to influence 5-FU clearance, and female sex is reportedly associated with more frequent and severe toxicity [10]. Based on the increasing evidence of genetic components in inducing 5-FU toxicity, a pharmacogenomic profile has been conducted in 2 patients who experienced 5-FU adverse reactions, studying the more frequent polymorphisms in the above-mentioned genes. In particular, we investigated the: (1) variability in the number of repetitions (2 or 3) of a sequence of 28 bases present in the 50 untranslated region of the TS gene (TSER 28-bp variable number tandem repeat, VNTR; TSER: TS enhancer region) [11, 12]; (2) presence of single-nucleotide polymorphisms in the MTHFR gene that result in the modification of MTHFR activity; in particular, the 677C→T polymorphism induces a change in codon 222 (Ala222Val) [13, 14], while the 1298A→C polymorphism induces the codon substitution Glu429Ala [15] – both these polymorphisms have been linked to an increased 5-FU chemosensitivity [16–18], and (3) hepatic enzyme dihydroprymidine dehydrogenase (DPYD) which catabolizes 5-FU, playing a primary role in determining the 5-FU half-life and the plasma concentration. The gene codifying for DPYD may present an mRNA splicing error at the level of intron 14 (IV14 + 1 G→A), associated with the production of a nonfunctional enzyme [19, 20], resulting in serious and potentially fatal adverse reactions if 5-FU is administered.

### Patients and Methods

#### Patients

Two patients, both of Caucasian ethnicity, with a diagnosis of sinus adenoid cystic carcinoma stage IV (patient A) and moderately poorly differentiated colon adenocarcinoma (patient B), were referred to our Division of Oncology. A chemotherapy regimen that included 5-FU was prescribed for both patients. After a few cycles, they experienced severe 5-FU-related toxicity. During the normal routine blood sample collection, a blood aliquot was taken for the genetic studies of the 5-FU target gene polymorphisms and was frozen until use. Informed consent was obtained from both patients.

#### DNA Extraction

The DNA extraction was conducted as suggested by the manufacturer (Qiagen Italia, Milano, Italy). Briefly, an aliquot (200 μl) of blood was incubated at 56°C for 10 min with the Buffer AL (Qiagen Italia) in the presence of 20 μl proteinase K (≥600 mAU/ml; Qiagen Italia) and 4 μl RNase A (100 mg/ml; Promega Italia, Milano, Italy). Then, 200 μl ethanol 100% was added, the mixture was applied to the QIAamp Mini spin column and washed with different buffers, given by the manufacturer. After the last wash, the column was eluted with 100 μl Buffer AE (Qiagen Italia) and stored in 5 μg aliquots at −20°C.

#### Real-Time PCR

RT-PCR was performed to amplify the following genetic markers: MTHFR C677T, MTHFR A1298C and DPYD IVS14 + 1 G→A, using the kit ‘fluoropyrimidine response’ (Diatech, Jesi, Italy), according to the manufacturer instructions. The temperature profile was 95°C for 1 min, 95°C for 15 s/54°C for 30 s/72°C for 20 s, 35 cycles; 60°C for 5 min. The signal was acquired from the green channel. Samples were then analyzed in the Pyrosequencing System (Diatech). Sequences analyzed were: MTHFR C677T: C/TC-ACTGGTGTG; MTHFR A1298C: TT/GCTCAGCTGGT; DPYD IVS14 + 1 G→A: CAAAG/ATAAGTGTG.

#### End-Point Amplification TSER and Gel Electrophoresis

In order to amplify the TSER marker, the PCR reaction was set up according to the ‘fluoropyrimidine response’ kit instructions. RT-PCR amplification was conducted at the following temperatures: 95°C for 1 min; 35 cycles of 95°C for 20 s/60°C for 10 s/72°C for 10 s; 72°C for 5 min. PCR products were subjected to electrophoresis on 2.2% agarose gel, visualized by ethidium bromide staining and photographed under UV light.

### Results

Patient A, a female aged 21 years, was diagnosed with left maxillary sinus adenoid cystic carcinoma stage IV, T4aM1 in July 2009. The patient underwent a surgical procedure, and in February 2010, the CT scan revealed a lung and a suspected liver progression of disease. A chemotherapy approach was then decided, and chemotherapy with cisplatin (25 mg/m2, days 1–4) plus 5-FU (750
mg/m², days 1–4) in continuous infusion was performed. After treatment conclusion, a grade 4 (G4) oral mucositis developed, with thrombocytopenia, anemia and neutropenia G4, lasting more than 10 days.

Patient B, a male aged 30 years, was diagnosed in July 2006 with a moderately poorly differentiated adenocarcinoma in the sigmoid colon, wholly infiltrating the bowel wall, extending to the pericolic adipose tissue (pT3 pN0/10 cM0, Dukes’ stage B or Astler and Coller stage B2). An adjuvant chemotherapy with FOLFOX (5-FU, oxaliplatin, folinic acid) was started in September 2006. During the prescribed cycles of chemotherapy, the patient experienced oxaliplatin-induced neurotoxicity, liver toxicity, nausea, vomiting and stomatitis G2. Despite this, the patient was able to complete the chemotherapy program of 12 FOLFOX cycles. After 30 months, the patient underwent thoracic surgery with a diagnosis of metastatic adenocarcinoma of colorectal origin. Later, the patient repeated the FOLFOX-6 scheme, but on the first day, uncontrollable vomiting and diarrhea occurred, due, according to clinicians, to G2 mucositis. Based on the positive anamnesis for 5-FU adverse reactions, such as stomatitis and hepatotoxicity, and on the symptoms observed in the new chemotherapeutic treatment, clinicians diagnosed a 5-FU adverse reaction. Thus, the systemic toxicity of the 2 patients was G4 mucositis and pancytopenia in patient A, while patient B developed G3–4 diarrhea, G2 mucositis and hepatotoxicity.

The tandem repeat sequences identified in the promoter of another 5-FU target gene, the TS enzyme, is involved in the 5-FU clinical response. Indeed, it was shown that patients possessing the 2R DNA was extracted as described and polymorphisms were detected using the pyrosequencing system, as described in Patients and Methods.

### Discussion

The prediction of response or toxicity and therapy individualization are becoming very important tools in cancer chemotherapy. There have been, indeed, numerous studies on the relationship between genotypes and the response to chemotherapeutic agents [21]. Potentially useful pharmacogenomic markers of the response to chemotherapeutic agents are now available. Here, using a commercially available kit, we reported 2 patients with severe systemic toxicity following 5-FU therapy that may be linked to polymorphisms found in the MTHFR and TSER genes. Both the MTHFR polymorphisms studied are associated with a reduced enzyme activity [22]. It has been suggested that the decreased MTHFR enzymatic activity may increase the 5-FU cytotoxic activity, due to a high level of intracellular 5,10-MTHF concentration, enhancing the TS inhibition [22]. Accordingly, tumor cell lines carrying C677T or A1298C mutations on the MTHFR gene show higher sensitivity to 5-FU exposure [23, 24]. However, results obtained from clinical studies are inconclusive on the role of the C677T variant on toxic side effects of 5-FU therapy. In contrast, the A1298C genotype in advanced colorectal cancer has been correlated with an increased risk of developing severe 5-FU adverse reactions (reviewed in De Mattia and Toffoli [22]), although mechanisms underlying this phenomenon have not yet been identified. Both patients described here are heterozygous for the MTHFR A1298C allele, and this gene profile may be responsible, at least in part, for the clinical findings. Furthermore, patient B, who expressed the C677T polymorphism, developed severe diarrhea, in line with previous published clinical findings in patients affected by metastatic colorectal cancer [8].

The tandem repeat sequences identified in the promoter of another 5-FU target gene, the TS enzyme, is involved in the 5-FU clinical response. Indeed, it was shown that the TS promoter could be polymorphic, and one of the polymorphisms influences the translation efficiency of TS. The TS promoter expresses a 28-bp tandem repeat, presented either as a double tandem repeat (2R) or triple tandem repeat (3R). The triple 28-bp repeats (3R) are associated with higher expression of both TS mRNA and protein compared to the double 28-bp repeats [11, 12]. It has been demonstrated that patients possessing the 2R

### Table 1. Polymorphisms in the 5-FU genes reported in 2 patients who underwent 5-FU therapy

<table>
<thead>
<tr>
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<th>Patient A</th>
<th>Patient B</th>
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<tbody>
<tr>
<td>MTHFR C677T</td>
<td>wild type (C/C)</td>
<td>heterozygous (C/T)</td>
</tr>
<tr>
<td>MTHFR A1298C</td>
<td>heterozygous (A/C)</td>
<td>heterozygous (A/C)</td>
</tr>
<tr>
<td>DPYD IVS14 + 1 G→A</td>
<td>wild type (G/G)</td>
<td>wild type (G/G)</td>
</tr>
<tr>
<td>TSER 28-bp VNTR repeats</td>
<td>2R/3R repeats</td>
<td>2R/2R repeats</td>
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DNA was extracted as described and polymorphisms were detected using the pyrosequencing system, as described in Patients and Methods.
variant allele show a significantly higher risk of severe toxicity to chemotherapy, and the risk of toxicity significantly increased with the number of 2R alleles [25, 26]. The rationale of this observation is that the 2R/2R genotype, giving rise to a low copy number of TS, did not protect normal cells against the 5-FU-induced toxicity.

Thus, with patient A being homozygous 2R/2R and patient B heterozygous 2R/3R, our patients were both exposed to a higher risk of 5-FU-induced toxicity.

Taken together, the 5-FU gene profile of our patients strongly suggested that polymorphisms present in the target genes examined may contribute to the adverse effects seen during the 5-FU therapy. To what extent these polymorphisms induced the adverse effects cannot be established at present; however, our results strengthen the relevance of the 5-FU-related pharmacogenomic profile to predict the response outcome and the chemotherapy toxicity in patients treated with this drug.

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