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Research Article

Analysis of the Thymidylate Synthase Gene Structure in Colorectal Cancer Patients and Its Possible Relation with the 5-Fluorouracil Drug Response

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Thymidylate synthase (TS) catalyzes methylation of dUMP to dTMP and it is the target for the 5-Fluorouracil (5-FU) activity. Barbour et al. showed that variant structural forms of TS in tumour cell lines confer resistance to fluoropyrimidines. We planned to perform the whole TS gene structure by means of sequencing techniques in human colorectal cancer (CRC) samples to try to identify the presence of any possible TS variant form that could be responsible of fluoropyrimidines drug resistance and of the worse prognosis. We performed the TS-DNA gene sequence in 68 CRC from patients of A, B, and C Dukes' stages and different histological grade, but we did not find any mutation in the TS-DNA structure. In the future we intend to widen the TS structure analysis to the metastatic CRCs, because due to their higher genomic instability, they could present a TS variant form responsible of the fluoropyrimidines drug resistance and the worse prognosis.

1. Introduction

TS is a key enzyme in the *de novo* synthesis of dTMP, an essential precursor of DNA, which catalyses the methylation of dUMP to dTMP [1].

The critical role of TS in the nucleotide metabolism has made it an important target of a variety of chemotherapeutic agents including 5-FU, 5-FU prodrugs as capecitabine, and novel folate-based TS inhibitor such as raltitrexed and pemetrexed, used in the treatment of colorectal and other solid tumours [2]. Resistance to fluoropyrimidines and other TS inhibitors may occur through a variety of mechanisms including elevated intracellular TS levels resulting from increases in TS transcription [3] and translation [4].

In CRC patients the TS intratumoural expression may predict for the sensitivity to 5-FU and other TS inhibitor-based chemotherapies [5, 6] and may also be an important prognostic marker [7]. High TS expression in early stage CRC patients seems to predict for poorer overall survival in both chemotherapy-treated and untreated patients following surgery [4]. Also, metastatic CRC patients with high TS levels are unlikely to respond to infusional treatment with 5-FU, whereas patients with low TS levels have higher than average response rate [8, 9]. In a previous study, however, we demonstrated that higher TS levels are favourable prognostic factors for disease free and overall survival [10]. To date the real value of the TS level as prognostic factor is doubtful and some authors are in disagreement about it [11]. The TS gene, containing 7 coding exons, is located in chromosome 18q, for which a high percentage of monosomic loss has been reported to be cell-cycle dependent, although some recent evidence points are more oriented towards proliferation-dependence. It can be predicted that mutations in the TS gene might result in a modification of its structure and then in its ability to interact with the fluoropyrimidines. This prediction has been born out in a variety of studies

Table 1: Clinical features of patients

No. of patients	68
Median age	67
Sex	
Male	30
Female	38

Table 2: Site of primary tumour.

Right colon	21
Left colon	34
Rectum	13

Table 3: Tumour staging and grading.

Tumour staging	
Dukes A	10
Dukes B	27
Dukes C	31
Grading	
G1	7
G2	36
G3	25

Table 4: Primer sequences.

Prim name	Primer sequence
1 F	CGCCGCGCCACTTCGC
1 R	CTCCCCAGCCGCGCCTCC
2 F	GGATGGCATGATCTGTC
2 R	CTGCTGTGTTGAGAACAG
3 F	CAACTGAGATGGCTTAAG
3 R	GCAAACACGTGCTAGGAAGG
4 F	GCCATCTCATGACATG
4 R	CCCTCAGTGCCTCTGCAC
5 F	CTTTGCCTTTAGCTGTG
5 R	GAGCTCATGTGGTAGGCT
6 F	GCGGTGTCTGCATATT
6 R	GCATTGAGCAGATACCTG
7 F	CACGGACATGAGGAGC
7 R	CTAAAGACTGACAATATC

[12–15]. S. H. Berger and F. G. Berger reported that the human colon tumour cell line HCT116 produces a variant structural form of TS, in addition to the common form found in all colon cell lines tested. Among the TS overproduction derivatives of HCT116, cells overexposing the novel forms are more resistant to FdUrd, compared with cells overexpressing the normal form [15]. So, an association between drug response and altered TS structure could suggest that the novel TS form, which is encoded by a variant structural TS gene, confers relative resistance to FdUrd [1]; this was supported by preliminary kinetic data indicating that this novel form has a reduced affinity for FdUMP [16]. This variant form presents a replacement of an evolutionary conserved tyrosine by a histidine at residue 33 of the TS polypeptide [17, 18]; this mutation represents the only difference between the two TS forms and must account for the structural and functional differences between them. In an our previous paper we performed the analysis of the TS structure in patients bearing CRC to try to demonstrate the presence of that specific mutation, but we did not find it in any patient [19]. Here, we intend to proceed on the use of sequencing techniques to see if any TS variant form could be present in human cancer samples from patients who underwent surgery for primary colorectal cancer and previously untreated and try to find relationship between any hypothetical TS variant form with the 5-FU treatment.

We performed the TS-DNA gene sequence in 68 cancer samples from patients of different Dukes' stages (A, B, and C) and histological grade but we did not find any change in the TS-DNA structure.

2. Materials and Methods

2.1. Patients. TS structure was assessed in a series of 68 patients who underwent surgery for primary colorectal carcinoma confirmed histologically and previously untreated. The following exclusion criteria were applied: history of other neoplasias apart of CRC and death occurring within 30 days following surgery and due to postoperative complications. The tumours were staged and graded according Dukes' system and their clinicopathological features were summarised in Tables 1, 2, and 3.

2.2. Colorectal Tumour Samples. Primary tumour and corresponding colonic mucosa were obtained with informed consent from patients. The samples at surgery were divided in two parts: one was frozen at −80° until analysis, and the other portion was embedded in paraffin to confirm histologically the absence of contamination by normal and necrotic tissue and lymphocytes.

2.3. Extraction of Genomic DNA, PCR Analyses, and Sequencing. Genomic DNA was extracted from tumour and mucosa samples with a commercial Kit purchased from Qiagen (Kjvenlo, The Netherlands) and stored at −20°.

The TS gene of tumour and mucosa samples was amplified in seven different PCR reactions using DNA primers of 16–20 bases in length placed in the adjacent intronic regions of exons 1, 2, 3, 4, 5, 6, and 7 and listed in Table 4.

Amplification was performed using a Perkin Elmer model 2400 thermal cycler (Boston USA). Reaction mixture included 5 μL of 10% dimethylsulfoxide, 5 μL of 10 × buffer, 1.5 μL of 1.5 mM $MgCl_2$, 1 μL of 10 mM dNTPs, 200–500 ng of genomic DNA, 200 pmol of both upstream and downstream primers, and 1 Units of Taq DNA polymerase, in a final volume of 50 μL. Amplification was run for 35 cycles with each cycle consisting in a denaturation step at 95°C for 1 minutes, a primer annealing step at 51°C for 1 minute, and an extention step at 72°C for 2 minutes. PCR was terminated by incubation at 72°C for 10 minutes.

The length of the amplification products ranged from 180 to 310 bases. Amplified fragments were analysed by agarose gel and visualized by ethidium bromide staining. DNA samples, generated with independently repeated PCR products, were sequenced with the SequiTherm EXCEL II DNA sequencing kit (Epicenter, Madison, WI) on a LI-COR 4000 (MWG-Biotech, Ebersberg, Germany) sequencer.

3. Results

The enzyme structure obtained by sequencing the TS genomic DNA of each sample was performed in 68 patients with operable CRC, untreated with previous chemotherapy. Primary tumours and corresponding colonic mucosa were obtained from each patient and the TS genomic structure was performed on each of them, after histological control. 7 patients had G1 histological grade, 36 had G2, and 25 had G3 histological grade; 10 patients had Dukes' A tumours, 27 patients had Dukes' B, and 31 patients had Dukes C cancers. The median age of patients was 67 years; 30 patients were male and 38 female. In all the tumour samples evaluated we did not find any TS-DNA variant structure in tumour: all A, B, and C Dukes' patients showed stable TS-DNA, and also in the germline genome no TS-DNA variants have been observed.

4. Discussion

The structure of the macromolecular target of a cytotoxic drug is a critical determinant of cellular sensitivity to that drug. Recently, TS has become the subject of several studies aimed to elucidate a possible "clinical" role of TS detection either as determinant of drug resistance or as prognostic marker of the disease and predictive factor of the treatment.

Many studies are currently examining the real value of TS expression levels as a prognostic factor, but in the mean time significant uncertainty prevails; whatever its prognostic influence, to date no studies have been able to establish a "cut-off" that is important for clinical usage. On the whole, all of these in vitro studies have promising implications in the study of the role of TS in clinical practice.

Moreover, the unclear meaning of "levels" not only as a prognostic indicator but also as marker predictive of resistance could be due to mechanisms different from over expression, as mutation.

In fact some authors focused their attention on the TS structure with a double aim: development of a new classes of TS-inhibitors with a different mechanism of action and generation of TS mutants to develop gene-therapy strategies. Berger and coworkers showed that a single naturally occurring change, a Tyr to His replacement, in the primary structure of the TS molecule confers relative resistance to the TS-directed antimetabolite FdUrd in HCT 116 cells. The Tyr to His mutation is the only difference between the altered form of TS and the normal form and, therefore, must be responsible for the diminished effectiveness of the enzyme as a drug target.

The data in those studies, together with previously published experiments, strongly favour the notion that the Tyr to His' mutation is responsible for the relative FdUrd resistance of cell line HCT 116 [15, 16]. However, as noted earlier, it is quite possible that factors in addition to this mutation contribute to the phenotype of HCT116 and other colon cell lines. The frequency of the Tyr33 to His33 mutation in the normal and in the pathological human populations is unknown. The altered TS may exist as a polymorphism in humans [16]; alternatively, it may have spontaneously arisen during tumorigenesis or during establishment of the cell lines in culture.

Distinguishing among these possibilities will be of great utility in assessing whether variant forms of TS identified in cultured cell lines are segregating in the human population and have an impact upon clinical response to 5-fluorouracil therapy in cancer patients. On the basis of this knowledge we hoped to see if in human CRC samples it could be possible to find the same Tyr33 to His33 substitution in the TS structure and eventually to correlate this finding with some clinicopathological parameters such as age and sex, tumour size and location, histological grade, Dukes' stage, 5-FU and Raltitrexed treatment, and disease free and overall survivals, to see if that point mutation could be considered a reliable marker of drug resistance and of prognosis. In those samples we did not find that point mutation at the codon 33 [19].

In this study we intend to proceed on the use of sequencing techniques to see if any TS variant form could be present in human cancer samples from patients who underwent surgery for primary colorectal cancer (CRC) and previously untreated and try to find a relationship between any hypothetical TS variant form with the 5-FU treatment and prognosis.

We performed the TS-DNA gene sequence in 68 cancer samples from patients of different Dukes' stages (A, B, and C) and histological grade, but we did not find any mutation in the TS-DNA structure. The conclusions that could be drown are that in Dukes' A, B, and C CRCs there are no changes in the TS-DNA gene structure and that the evaluation of the TS expression is the main CRC prognostic and drug response marker. What remains is to evaluate the TS gene structure of the D metastatic Dukes' CRCs: in these tumours it might be possible to find TS-DNA structural changes related with their higher genomic instability and this fact could give an explanation of the 5-FU drug resistance and worse prognosis.

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