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ORIGINAL ARTICLE

Thymidylate synthase polymorphism in Mexican patients with colon cancer treated with 5-fluorouracil

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Summary

Purpose: *We analyzed the genotype and allele frequency of variable number tandem repeats (VNTR)-thymidylate synthase (TS) and its relationship with the disease evolution in colon cancer patients.*

Methods: *We selected 24 paraffin-embedded colon cancer tissue samples from Mexican patients who received a 5-fluorouracil (5-FU)-based chemotherapy regimen. Tumor tissue was digested with proteinase K and genomic DNA was isolated by the standard method with phenol-chloroform extraction. Polymerase chain reaction (PCR) was performed for TS genotyping of VNTR and the results were evaluated directly in a stained agarose gel.*

Results: *The allele frequency of 2 repeats (2R) was greater (0.66) than 3R (0.34) in metastatic colon cancer (x^2=10.24; p=0.001); however, no difference in allelic distribution between 2R (0.54) and 3R (0.46) in non metastatic patients was observed (x^2=0.640; p=0.424).*

Conclusion: *Our results suggest that Mexican patients with colon cancer present differences in the allelic distribution, the 2R allele being the most frequent.*

Key words: *colon cancer, 5-fluorouracil, pharmacogenetics, thymidylate synthase, VNTR*

Introduction

In Mexico, colon cancer represents 3.8% of new cancer cases [1]. Its incidence is higher in the Mexican northern states, which, despite their people having the highest income among Mexican states in general, they have non healthy dietary habits (high meat and animal fat consumption and low intake of vegetable fibers) in comparison with the southern states' population [2,3].

Chemotherapy is essential to provide a chance for cure or increase the survival of patients with advanced disease, with 5-FU plus leucovorin (5-FU/LV) being the regimen most patients are administered [4]. Pharmacogenetics allows understanding of the association between genetic variations and drug response [5,6]. Genetic variability implies kinetic differences among those enzymes devoted to metabolize drugs. Therefore, genetic variability of these enzymes influences toxicity of

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anticancer agents and individual response to chemotherapy [7].

The most important target of 5-FU is the enzyme TS [8]. TS catalyzes the reductive methylation of dUMP by 5, 10-methylene tetrahydrofolate, producing dTMP and dihydrofolate [9]. The 5' untranslated region (5'-UTR) of the TS gene contains variable number of VNTR, which consists of 2R or 3R of a 28-bp sequences (An R sequence is as follows: CCGCGCCACTTGGCCTGCCTCCGTCCCG) [10,11]. Some researchers have suggested a poorer response to 5-FU in 3R-TS homozygous patients compared with 2R-TS homozygous patients (2R/2R) [12]. 2R/2R patients show lower TS mRNA levels and a significantly better response to 5-FU compared with patients homozygous for 3R/3R (50% 2R/2R vs 9% 3R/3R; p=0.04) [13].

Moreover, ethnicity difference of the TS genotype has been reported [14]. Homozygous triple repeat subjects are twice as common in Chinese subjects (67%) than in Caucasian subjects (38%). This significant ethnic variation in the TS gene may have a significant impact on drug therapy [9]. Therefore, it is valuable for our population to analyze the TS genotype for predicting response to 5-FU treatment in patients with colon cancer.

The purpose of this study was to analyze the genotype and allele frequency of VNTR-TS and their relationship with the evolution of colon cancer patients.

Methods

Patients and samples

Twenty-nine tissue samples of colon cancer were used, which were previously fixed with formalin and embedded in paraffin. The above size sample was estimated according to the number of new colon cancer cases reported yearly by the National Center for Epidemiologic Surveillance and Disease Control of Mexico (http://portal.salud.gob.mx/contenidos/tramites/cenavece.html). The patients attended the Center Against Cancer Unity Hospital "José Eleuterio González, Autonomous University of Nuevo Leon (UANL). Medical data used in this study (chemotherapy type, chemotherapy cycle number, sex, age, response to treatment, and time to progression) were retrieved from the patient files. Patients were treated with surgery, followed by different chemotherapy regimens, including 5-FU in most cases: Mayo clinic (5-FU plus leucovorin), FOLFOX (oxaliplatin plus leucovorin plus 5-FU) or XELOX (oxaliplatin plus capecitabine). One patient was treated with surgery alone. The study group was divided into metastatic- (for assessment of treatment response) and non metastatic patients (for evaluation of recurrence or disease progression). Patients were retrospectively followed for at least one year after chemotherapy. Approval for this project was obtained from the Medical School Ethics Committee of the Autonomous University of Nuevo Leon: BI 10-003.

The new guideline for response evaluation criteria for solid tumors (RECIST, URL: http://www.ncbi.nlm.nih.gov/pubmed/19097774) was used. This guideline proposes to measure the diameter of the largest lesions and their total sum. These criteria consider two types of tumors: measurable lesions with computerized tomography (those with a diameter greater than 1.0 cm) and those non-measurable lesions (with diameters less than 1.0 cm) [15]. In the evaluation of treatment response, three different situations could be distinguished: complete response (CR), showing disappearance of all lesions; partial response (PR), presenting a reduction ≥ 30%, equivalent to the diameter sum of all largest lesions; or a stable disease (SD), which does not meet PR or CR criteria.

PCR amplification

Tissue was digested with proteinase K and DNA was isolated using standard procedures. PCR amplification of TS VNTRs was carried out using the primers (Invitrogen, Massachusetts, USA) forward 5´-GAAAAGGCGCGCGGAAGGGGTCC-3´ and reverse 5´-TCCGAGCCGGCCACAGGCAT-3´ [10]. The PCR product was separated on 12% polyacrylamide gel and visualized with ethidium bromide (UVP, CA, USA).

Statistics

Demographic variables were assessed with non-parametric analysis (median, quartile: Q, x^2). Genotypic and allelic frequencies were evaluated by x^2 and binomial square ($p2+2pq+q2=1$), respectively. $P < 0.05$ was considered significant. Statistical calculations were performed using the SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA).

Results

A total of 29 medical files of the University Center Against Cancer of the "José Eleuterio González" University Hospital (UANL), were reviewed to collect the clinicopathologic patient in-

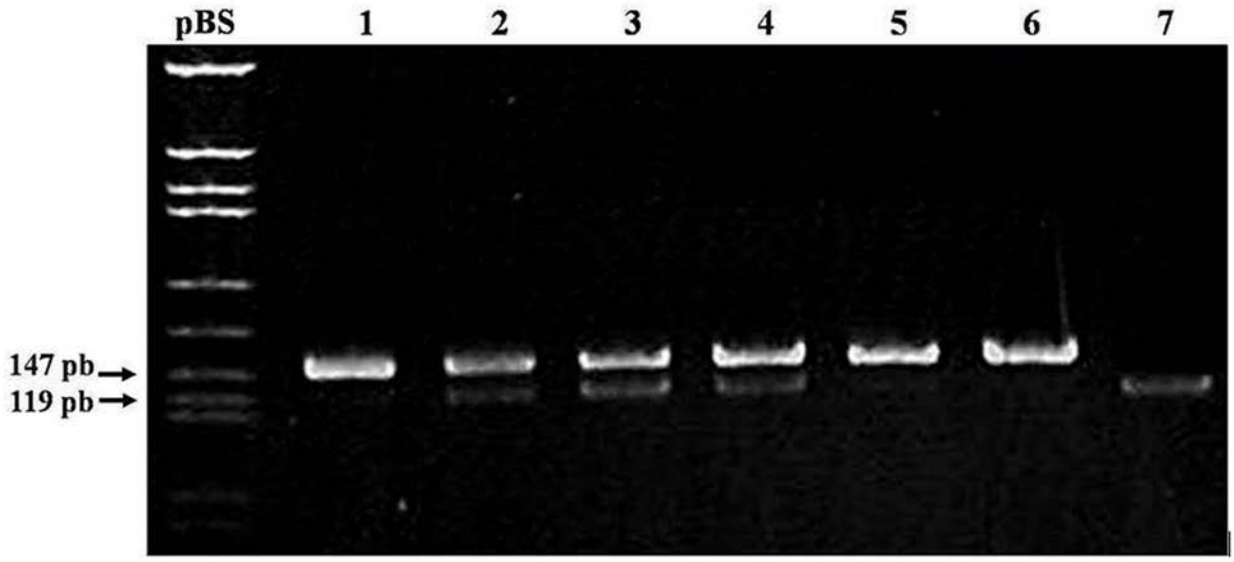

Figure 1. Genotypes-based on VNTR-TS, determined by PCR. The amplified products were resolved by polyacrylamide gel electrophoresis (PAGE) in a 12- polyacrylamide gel. Homozygous for 2R/2R: 119 bp (line 7). Heterozygous 2R/3R: 147bp and 119bp (lines 2-4). Homozygous 3R/3R: 147bp (lines 1,5-6). Homozygous 2R/2R patients showed a better response to 5-FU compared with those homozygous 3R/3R.

formation. The patient median age was 63 years (Q1= 52; Q3= 69). Of all patients (N=29) 62% were male and 38% female.

Of the 29 samples, 12 were metastatic and 17 non-metastatic.

Of the 12 metastatic patients, 10 received chemotherapy and 2 were treated with surgical resection alone; of the 10 patients treated with chemotherapy, one was excluded due to lack of follow-up, leaving thus 9 patients eligible to assess treatment response.

Treatment response was assessed in 88.9% (N=8) patients and showed 3 CRs, 3 PRs and 2 SDs. No response was observed in only one case. Five (62.5%) patients experienced disease progression. The average time to progression was 12 ±3 months and only 2 (25%) patients progressed before 12 months. Of the 17 non-metastatic patients, 15 received adjuvant chemotherapy and only 1 experienced disease recurrence.

The genotypes of TS based on VNTRs were determined by PCR and amplified fragments are shown in Figure 1. The 24 samples analyzed were distributed as follows: 2R/2R: 11 (37.93%), 2R/3R: 12 (41.38%) and 3R/3R: 6 (20.69%). 2R allele was the most frequent (59%) and 3R was present in 41% of the patients.

Medical data and genotypic results of colon cancer cases were obtained. After one year of patients' monitoring, we noted two clearly

Table 1. Medical data and genotypic results* of 9 metastatic colon cancer cases

Chemo-therapy	*Gender*	*Age (years)*	*Cycles (number)*	*Response*	*Progres-sion*	*Time to progression (months)*	*Genotype*	*x^2, p*	*Allele*	*x^2, p*
Mayo	M	56	6	CR	No		2R/2R	0.333, 0.564	2R (0.83)	43.56,*$4.11x10^{-11}$
Mayo	M	46	8	CR	No		2R/3R		3R (0.17)	
FOLFOX	F	62	12	CR	No		2R/2R			
XELOX	M	57	8	PR	Yes	11	2R/2R	0.333, 0.564	2R (0.67)	11.56, *0.001
FOLFOX	M	51	12	PR	Yes	12	2R/2R		3R (0.33)	
Mayo	F	52	6	PR	No		3R/3R			
FOLFOX	F	66	6	SD	No		3R/3R	0.00, 1.00	2R (0.50)	0.00, 1.00
Mayo	M	55	6	SD	No		2R/2R		3R (0.50)	
FOLFOX	M	21	6	PD	Yes	6	2R/3R	NA	NA	NA

* Results of non parametric frequency analysis of genotypes-and 2R vs 3R
* Significant p values, M: males, F: females, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, NA: not analyzed

Table 2. Medical data and genotypic results of 15 non-metastatic colon cancer cases

Chemotherapy	*Sex*	*Age (years)*	*Cycle (numbers)*	*Recurrence*	*Genotype*	x^2, *p*	*Allele*	x^2, *p*
Mayo	M	49	3	No	2R/2R	9.14, 0.010	2R (0.50)	0.000, 1.000
Mayo	M	57	6	No	2R/3R		3R (0.50)	
Mayo	M	59	6	No	2R/3G			
Mayo	F	62	3	No	2R/3G			
Mayo	M	63	6	No	2R/3G			
Mayo	M	65	6	No	2R/3G			
Mayo	F	65	6	No	2R/3G			
Mayo	M	68	2	No	2R/3G			
Mayo	M	69	6	No	3R/3R			
Mayo	M	77	6	No	3R/3R			
Mayo	F	78	6	No	2R/3G			
Mayo	F	87	6	No	2R/3G			
FOLFOX	F	62	6	No	2R/3R			
Mayo	F	76	12	No	2R/2R			
FOLFOX	F	76	6	Yes	2R/2R	NA	NA	NA

M: male, F: female

differentiated groups. One group consisted of patients with metastatic colon cancer and another group was formed by patients without metastases. Table 1 shows that, according to patient response, a group formed by 9 metastatic patients was divided into 4 subgroups: the subgroup 1 consisted of 3 asymptomatic CRs. These patients showed the following allelic distribution: 2R:3R (0.83:0.17; $p=4.11x10^{-11}$). The subgroup 2 was formed by 3 PRs (0.67:0.33;p=0.001). The subgroup 3 was formed by 2 patients showing SD (0.50:0.50;p= 1.00). And the subgroup 4 was formed by 1 patient showing disease progression (0.50:0.50). Table 2 shows the individual medical data and results of genotyping of a group constituted by 14 patients. None of these patients showed cancer recurrence. Their allelic distribution was 2R: 3R, with a frequency 0.50:0.50 (p=1.000).

Discussion

With the progress in molecular biology and the human genome sequencing, a new area in pharmacology has emerged: pharmacogenetics, which in oncology aims to personalize chemotherapy of practically any condition, therapy based on a specific genotype in order to increase the probability of improving treatment outcome and identify predictive factors to choose the best drug based on tolerability and efficacy [16-18].

In this study, we genotyped TS VNTRs located within the 5´ UTR of the sequence [19]. The study group consisted of 24 paraffin-embedded tissue samples from colon cancer patients treated postoperatively with different chemotherapy regimens based on 5-FU in most of the cases and in patients treated with surgery alone.

In the metastatic group, three patients achieved CR to treatment, of which two had a homozygous 2R/2R genotype and one a heterozygous 2R/3R genotype; none of these patients had PD during the follow up period. Three patients had PR, of which two had a homozygous 2R/2R genotype and one had a homozygous 3R/3R genotype. All of these patients showed PD in a median time of 12 months. Only two patients had SD, one with homozygous 2R/2R genotype and the other with homozygous 3R/3R genotype.

Some researchers have reported that TS is not a good biomarker to define a pharmacological response to 5-FU in the studied population [20] and a research group in the Netherlands reported a low correlation between the TM protein expression by immunohistochemistry and TS activity, proposing that genotyping is more predictive for therapy response [21]. This could be attributed to ethnic characteristics of the studied population [22].

Conversely, genotyping and quantification of TS expression levels in Chinese populations is considered as a potential biomarker of response to therapy with 5-FU [23]. Furthermore, Xi et al. [24] observed a clear correlation between genotype and phenotype after chemotherapy with fluoropyrimidines and pointed out that a 2R/2R genotype in patients with colon cancer predicts a better re-

sponse to their treatment probably because these patients had a good correlation between 2R/2R genotype with an adequate metabolism of 5-FU [13,25,26]. This controversy could possibly be attributed to ethnic characteristics of the studied population [25].

TS is the main enzyme targeting 5-FU; however, dihydropyrimidine dehydrogenase (DPD) and metylentetrahydrofolate reductase (MTHFR), together with TS, predict 100% of a good response to 5-FU [27-29]. Ideally, the mutations associated with the response to 5-FU in these three genes should be genotyped to define a more specific response profile and provide a better treatment scheme [30,31].

In 2003, Mandola et al. discovered that proteins from upstream transcription factor 1 (USF-1) bind to the 5´ UTR region of TS and increase the transcription of the gene [10]. On the other hand, Sp-1 transcription factor, also known as specificity protein 1, binds to GC motifs in TS VNTRs, acting as transcription activator [32]. The above facts support the proposal of Hassan et al. [33] who reported that USF-1 and Sp-1 could be considered as therapeutic targets that directly modulate TS expression.

This study represents the first analysis of the VNTR genotype-TS in Mexican patients with colon cancer. In conclusion, the population of this study showed a higher frequency of 2A allele, which was associated with better response to 5-FU treatment. However, further studies with larger sample sizes are needed in our population to analyze the genotype distribution and its association with treatment response with greater accuracy.

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Conflict of interests

The authors declare no confict of interests.

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