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RESEARCH

TWIST1 Promoter Methylation in Primary Colorectal Carcinoma

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Abstract TWIST1 gene, a transcription factor that belongs to the family of basic helix–loop–helix proteins, has been related to tumor progression and metastasis in different cancers. The aim of our study was to investigate TWIST1 promoter methylation in patients with primary colorectal carcinoma and determine its correlation with prognostic factors and disease outcome. Seventy-three patients with primary colorectal adenocarcinoma were studied. From each patient two tissue samples were collected: one sample of the tumor and one sample of normal colorectal tissue from an area located 15 cm away from the tumor. Samples of colorectal mucosa obtained from 30 individuals without malignant disease were also studied as a control group. All tissues were analyzed through methylation-specific PCR. TWIST1 hypermethylation was detected in colorectal specimens of 46 patients with cancer, but in none of the tissues from the nonmalignant control group ($p<0.001$). In cancer patients, TWIST1 hypermethylation was found in 38 of 73 tumor samples as compared with 20 of 73 matched samples of non-cancerous colorectal tissue ($P=0.001$). TWIST1 hypermethylation was not correlated with prognostic predictors for the disease outcome, patients' overall survival and disease-free survival rates. We concluded that TWIST1 hypermethylation is present in the colon and rectum of most patients with colorectal carcinoma, suggesting this molecular alteration may be involved in the process of colorectal carcinogenesis.

Keywords Colorectal cancer · *TWIST1* · Methylation · Prognosis · Colon

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Introduction

Hypermethylation of CpG dinucleotide "islands" located within promoter regions resulting in the silencing of tumor-suppressor genes is thought to be an important epigenetic mechanism for colorectal carcinogenesis [1, 2]. The so-called CpG island methylator phenotype (CIMP) is observed in approximately 30% of colorectal cancer cases and is characterized by the simultaneous methylation of multiple CpG islands in tumor DNA. This phenotype is more frequently observed in tumors with proximal location, microsatellite instability, and normal p53 [3–5].

Colorectal carcinomas have been shown to develop through a stepwise progression of several genetic and epigenetic events taking place during the well-described "adenoma-carcinoma" sequence [6, 7]. However, the identification of new molecular factors involved in progression of colorectal tumors is still a critical and necessary

step towards better understanding of colorectal carcinogenesis and development of new anticancer strategies.

In this context, TWIST1 has recently emerged as a potential cancer biomarker [8–10]. It is a highly conserved transcription factor that belongs to the family of basic helix–loop–helix proteins [11]. TWIST1 is implicated in lineage-specific cellular differentiation during early embryonic development through the regulation of mesodermal patterning, morphogenesis and osteogenesis. In cancer development, TWIST1 is thought to function as a prometastatic oncogene [12]. Expression of TWIST1 protein counteracts the proapoptotic effects of N-MYC by repression of p19ARF and thereby hampers p53 function. In addition, it has been shown to induce angiogenesis and chromosomal instability [13].

TWIST1 is often overexpressed in malignant tumors, and it is usually associated with poor prognosis [14–16]. Elevated levels of TWIST1 mRNA have been observed in different types of cancer, such as breast cancer [9, 17], diffuse-type gastric carcinomas [18, 19], esophageal squamous cell carcinoma [20], and pancreatic cancer [21]. More recently, Valde's-Mora et al. [15]. demonstrated that TWIST1 is significantly overexpressed in colorectal cancer samples as compared to normal colon mucosa, being significantly correlated with the presence of lymph node metastases. The authors propose TWIST1 as a new molecular marker of advanced malignancy and as a potential therapeutic target in colorectal cancer. The present study was designed to investigate TWIST1 promoter methylation in a series of patients with primary colorectal carcinoma and in controls without colorectal malignancies.

Materials and Methods

Patients

Seventy-three patients with histologically confirmed primary colorectal adenocarcinoma were prospectively enrolled in the study. There were 36 males and 37 females (mean age 64.1 years, range 39 to 75). Patients with diagnosis of familial adenomatous polyposis, hereditary nonpolyposis colon cancer or inflammatory bowel disease were excluded from the study. Other inclusion criteria were no prior surgery, radiation or cytotoxic therapy for the colorectal adenocarcinoma.

Pretreatment assessment included a complete medical history and physical examination, carcinoembryonic antigen measurement, colonoscopic examination, computed tomography of abdomen and pelvis and chest radiograph. After the surgical resection and histological examination of the specimen, the patient's TNM stage was determined [22]. The distribution was as follows: 11 patients stage I, 23 stage II, 27 stage III and 12 stage IV. Sixty-five tumors were histologically diagnosed as moderately differentiated and seven were diagnosed as poorly differentiated adenocarcinomas.

As a non-malignant control group, 30 patients submitted to colorectal resection for treatment of benign intestinal diseases were studied. This group included 14 males and 16 females (mean age 52.9 years, range 25 to 76) whose surgical specimens were histologically negative for malignant or pre-malignant lesions.

The present study was performed after approval by the Ethics and Scientific Committee of the Hospital de Clinicas de Porto Alegre, Federal University of Rio Grande do Sul. Informed consent was obtained from all patients before being enrolled in the study.

Tissues

All tissue samples were collected by the surgeons directly involved in the research right after removal of the surgical specimens. Two tissue samples were collected from each patient with colorectal carcinoma: one sample of the tumor and one sample of non-cancerous colorectal tissue from an area located 15 cm proximally to the tumor. Tissue samples from the non-malignant control group were obtained from a middle area of the resected colorectal specimen. All collected tissues were kept frozen until analysis.

Methylation-Specific PCR (MSP)

DNA to MSP analyses was isolated from frozen material using QIAamp DNA Mini Kit (Quiagen, Hilden, Germany). Extracted DNA was submitted to chemical treatment with sodium bissulfite as previously reported [23]. In brief, 10 μg of DNA was denatured by NaOH and modified by sodium bissulfite treatment of 16 h at 55°C. DNA was then purified using Wizard DNA CleanUp System (Promega, Madison, WI) according to the manufacturer's instructions, and ressuspended in 20 μl water and stored at 20°C until MSP analysis. Bissulflite- modified DNA quantification was made in device GeneQuantII (Amersham PharmaciaBiotech UK Ltd. England).

Promoter methylation status of *TWIST1* was analyzed by MSP as described previously [8]. For each reaction, 1 μl of sodium bissulfite-modified DNA was added to 24 μl of reaction buffer [1.25 mM dNTP, 16,6 mM $(NH4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0,1% DMSO and 1,25 units Taq DNA Polimerase] containing 10 ρmol of each primers pair to unmethylated (forward: 5′ TTTGGATGGGGTTGTTATTGT 3′; reverse: 5′ CCTAACCCAAACAACCAACC 3′) and

methylated (forward: 5′ TTTCGGATGGGGTTGTTATC 3′; reverse: 5′ AAACGACCTAACCCGAACG 3′) DNA sequences. These primers pairs were designed to amplify PCR products with 193 and 200 bp, respectively. PCR reactions were ′hot started` at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, with a final extension cycle of 72°C for 5 min. The MSP products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. The presence of a visible PCR product with 200 bp in length are predictive of methylated locus, while a 193 bp PCR product shows absence of methylation. CpG Universal methylated DNA (Qbiogene, Carlsbad, CA) was used as a positive control for methylated alleles and DNA from normal leucocytes were used as the negative control for unmethylated alleles. Negative controls without DNA were always included in all experiments.

Statistical Analysis

Methylation status and clinicopathological parameters were investigated by Chi-square test or Fisher's exact test where appropriate. Overall survival (OS) was defined as the time interval from diagnosis to death from any cause or, for patients remaining alive, the time interval from diagnosis to the last follow-up. Disease-free survival (DFS) was defined as the time interval from surgery to the time of disease progression or recurrence, to the last follow-up, or to death occurrence from any cause. To determine the association between TWIST1 hypermethylation and OS and DFS, we used the Log-rank test of Kaplan–Meier. Statistical significance was accepted at the 5% level.

Results

TWIST1 Promoter Methylation

In cancer patients, colorectal tissues were collected as paired samples of the tumor itself and of non-cancerous colorectal tissue from an area located 15 cm proximally to the tumor. TWIST1 hypermethylation was found in 38 of 73 tumor samples as compared with 20 of 73 matched proximal non-cancerous tissues ($p<0.001$). Either one of these results was statistically significant as compared with the analysis of the tissue samples from the non-malignant control group, in which no hypermethylation was detected ($p<0.001$). In 12 cancer patients TWIST1 hypermethylation was detected in both the tumor and the paired noncancerous colorectal tissue, in 26 cancer patients hypermethylation was detected only in the tumor and in eight patients hypermethylation was found only in the non-cancerous tissue sample.

Methylation Status and Clinicopathological Features of the Tumors

Clinical and pathological characteristics of the colorectal cancer cases were analyzed according to their methylation status (Table 1). TWIST1 hypermethylation was less frequently observed in tumors classified as TNM stage I as compared with tumors having more advanced stages ($p=0.04$). No significant differences were seen in TWIST1 hypermethylation with regard to age and gender of patients as well as with regard to degree of histological differentiation of the tumors.

After being submitted to surgical treatment, cancer patients were followed-up for a mean period of 40.23 month (range 1–63 months). During this period, 24 (33.3%) of the patients died (15 men and 9 women; mean age 67±12 years) and 18 (25%) suffered disease relapse. The overall survival (OS) and the disease-free survival (DSF) rate of these patients after four years were 60.5% and 46.5%, respectively. Univariate analysis was used to evaluate the influence of TWIST1 and other clinicopathological parameters on disease outcome (Table 2). It was observed that the advanced cancer stages negatively influence both OS and DFS ($p<0.01$), while male gender was correlated to reduction of DFS ($p=0.02$). In contrast, detection of TWIST1 hypermethylation within the tumor did not significantly influence the clinical course of these patients.

Discussion

Colorectal cancer is one of the most common malignancies throughout the world with more than one million new cases diagnosed each year. In the process of colorectal carcinogenesis, a series of tumor-suppressor genes such as *APC*, *p53*, and genes on chromosome 18q (*DCC*, *SMAD2*, and *DPC4/SMAD4*) are inactivated by mutations and chromosomal deletions [6]. A subset of colorectal tumors also shows a characteristic malignant phenotype which is associated with microsatellite instability (MSI) and inactivation of mismatch repair (MMR) genes such as *hMSH2* and *hMLH1* [24, 25]. Recently, CpG island (CGI) hypermethylation has been identified as a potentially important molecular factor in colorectal cancer [2, 3]. This alteration is usually associated with silencing of tumor-suppressor genes and absence of coding region mutation, serving as an alternative epigenetic mechanism in colorectal cancer development [26, 27].

In this context, the evaluation of relevant methylated genes might eventually become useful in identifying new potential targets for molecular detection of colorectal carcinoma, as even small amounts of methylated sequences are readily detectable by MSP.

Table 1 Association of *TWIST1* hypermethylation status and the main clinicopathological features in 73 CRC patients

Feature[a]	n	*TWIST1* hypermethylation status			
		Colorectal carcinomas		Paired nonneoplastic tissues	
		Methylated	P	Methylated	P
All patients	73	38		20	
Age (yrs)					
≥50	59	33	NS	17	NS
<50	14	05		03	
Gender					
Female	37	20	NS	11	NS
Male	36	18		09	
Tumor location					
Right colon	13	08	NS	0	0.042
Left colon	20	10		07	
Rectum	40	20		13	
Tumor differentiation					
Well/Moderate	65	31	NS	17	NS
Poor	7	06		03	
TNM stage					
I	11	02	0.04	02	NS
II	23	11		06	
III	27	18		10	
IV	12	07		02	

[a] Data missing for age, tumor differentiation and TNM tumor stage for one patient

TWIST1 has recently emerged as a cancer biomarker [8, 9, 15]. It has been identified as an oncogene involved in different oncogenic pathways such as inhibition of the p53-dependent apoptotic route [12], alteration of cell–cell adhesion mediated by the E-cadherin [28], and induction of epithelial-mesenchymal transition [29], increasing cell survival and metastatic capability.

The present study is the first to investigate the TWIST1 methylation status in patients with primary colorectal carcinoma as compared with controls without neoplasia. We were able to detect TWIST1 hypermethylation in colorectal specimens of 46 patients with cancer, but in none of the tissues from the nonmalignant control group ($p<0.01$). The absence hypermethylation in the control group suggests that this alteration is not merely incidental in colorectal carcinomas, but might rather represent a potential cofactor in development of the disease.

In patients with cancer, TWIST1 hypemethylation was more frequently seen in tumor tissues than in their paired non-neoplastic tissues, suggesting that TWIST1 might be considered a Type-C agent with the potential to be used as an epigenetic cancer biomarker [3]. The Type-C genes are characteristically hypermethylated in pre-invasive lesions and/or tumors and unmethylated in the mucosa of patients without cancer, being useful in early diagnosis of cancer. The presence of TWIST1 hypemethylation in a number of normal-appearing colorectal tissues obtained from patients with cancer suggests that it might be an early event in the process of colorectal carcinogenesis.

Our results are in line with the study conducted by Okada et al. [30], who investigated the association of *TWIST1* mRNA expression and *TWIST1* methylation with clinicopathologic features of patients with colorectal cancer. Those authors studied tumor specimens from 319 patients, corresponding normal colorectal nontumorous mucosa from 251 patients with cancer, and colorectal adenomas from 189 patients. They were able to find higher TWIST1 methylation level in colorectal adenoma and cancer than in normal colorectal mucosa. There was no correlation between TWIST1 methylation and TWIST1 expression. However, elevated TWIST1 expression was associated with unfavorable outcomes. During a mean follow-up of 46.7 months, the expression levels of TWIST1 in cancer specimens was 0.627 for patients who survived and 4.251 for the patients who died of cancer ($p=0.0019$). TWIST1 methylation was suggested as a useful biomarker for screening colorectal cancer and TWIST1 expression as potential prognostic predictor in patients with this type of malignancy.

Table 2 Impact of clinicopathological parameters and *TWIST1* hypermethylation on overall and disease-free survival in CRC patients

Feature	Overall survival		Disease-free survival	
	Median±standard deviation in months	P[a]	Median±standard deviation in months	P[a]
Age (yrs)				
≥50	46.7±2.96	NS	48.7±3.32	NS
<50	52.5±4.16		46.0±5.61	
Gender				
Female	49.3±3.45	NS	51.9±3.43	0.024
Male	45.9±3.60		42.8±4.45	
Tumor location				
Right colon	47.8±2.84	NS	49.4±3.16	NS
Left colon/rectum	47.9±5.11		43.8±6.26	
Histological differentiation				
Well/moderate	49.0±2.67	NS	50.0±3.96	NS
Poor	40.8±8.61		36.2±9.94	
TNM stage				
I	55.7±1.21	<0.0001	52.5±2.24	0.018
II	56.2±3.06		57.7±2.89	
III	45.1±4.17		37.1±5.37	
IV	23.4±5.73		-	
TWIST1 rmethylation in colorectal cancers				
Unmethylated	47.7±3.56	NS	47.7±3.84	NS
Methylated	47.3±3.45		47.9±4.15	
TWIST1 methylation in paired nonneoplastic tissue				
Unmethylated	49.7±3.02	NS	52.3±3.10	NS
Methylated	44.2±4.48		39.8±5.53	

[a] Log-rank test

We found a lower prevalence of TWIST1 hypemethylation in tumors classified as TNM stage I as compared with more advanced-stage tumors. However, we did not find a clear correlation between methylation and other standard prognostic parameters in colorectal cancer. Our data do not support the influence TWIST1 methylation in the clinical course of the disease as long-term follow-up of our patients failed to demonstrate an effect of TWIST1 hypemethylation in their OS and DFS. This observation, however, is somewhat confined by our sample size and needs further confirmation.

Reports on breast cancer also failed to demonstrate a correlation between TWIST1 methylation and prognostic factors for cancer outcome [9, 31]. Moreover, there seems to be no clear correlation between TWIST1 promoter methylation and TWIST1 protein or RNA expression [9]. This indicates that although TWIST1 CpG methylation is useful as an early biomarker in breast cancer diagnosis, there is no direct correlation with TWIST1 protein or RNA expression, which may be influenced by alternative compensatory molecular pathways. In the initial studies with colorectal cancer patients, the expression pattern of TWIST1 mRNA has been proposed as a potential prognostic marker of advanced malignancy, being associated with the presence of lymph node metastases as well as with unfavorable disease outcome [14].

Conclusions

We were able to provide evidence for an association of TWIST1 hypermethylation with colorectal cancer. Our data suggest that TWIST1 methylation might represent a new biomarker for this type of cancer. It should be pointed out, however, that this observation needs to be substantiated with additional studies analyzing a larger number of individuals and also the pathways involved this molecular mechanism. Further studies are warranted to elucidate the role of TWIST1 in colorectal carcinogenesis.

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