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ORIGINAL PAPERS

p16 gene methylation in colorectal cancer patients with long-term follow-up

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ABSTRACT

Introduction: *p16* gene plays an important role in the cell cycle regulation and is considered an important tumor suppressor gene. Several mechanisms of gene inactivation have been described; in this study we have focused on *p16* gene promoter methylation. In colorectal cancer *p16* gene methylation is a frequent event.

Methods: 326 patients with sporadic colorectal cancer were included. DNA was extracted from tumor tissue samples obtained during the surgical procedure. Promoter methylation was analyzed using bisulfite modification and was detected by quantitative methylation-specific PCR. Frequency of *p16* methylation was analyzed and compared with other clinicopathological variables.

Results: *p16* gene methylation was detected in 24,8% of patients. Methylation was associated with differentiation grade and with tumor location: methylation was frequent in poorly differentiated tumors and had low frequency in distal colon. The *p16* promoter methylation discriminated a subgroup of patients with better prognosis in poorly differentiated tumors.

Conclusions: *p16* methylation was a frequent event in our population and was able to induce differences in the overall survival of patients with poorly differentiated tumors.

Key words: *p16* gene. Methylation. Colorectal cancer. Prognosis.

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INTRODUCTION

Great efforts have been done to improve prediction of prognosis in colorectal patients (1-3). They have been focused on the genetic mechanisms implicated in tumorogenesis of colorectal cancer. The $p16^{INK4a}$ gene acts as a tumor suppressor gene and has associated with tumorogenesis when it is inactivated (4). This inactivation often occurs through promoter methylation. It is still unclear the prognosis influence of p16 in colorectal patients.

The *p16* gene is also known as CDKN2, MTS1, INK4a and CDK4I. It is implicated in the cell cycle control, playing an important role as tumor suppressor gene (4). This gene is located on region 9p21, comprised of 3 exons, and codes for a 16 kDa protein. *p16* inhibits the cyclin kinase D1-CDK4/6 complex, responsible for the phosphorylation of protein Rb, causing cell cycle arrest at G1 stage (5).

Alterations in the 9p21 gene region are frequent in human cancers (6). Several mechanisms for *p16* gene inactivation have been described: deletion, promoter methylation and point mutation, but their incidence depends on tumor type (7). Point mutations in *p16* are a rare event in human cancer and one of the most frequent causes for gene inactivation is loss of heterozygosity in 9p21 (8).

The CpG dinucleotides located in promoter region are called CpG islands. These regions are target for methylation as an important mechanism of transcriptional regulation (9,10). Methylation of promoter regions plays an important role in silencing of tumor suppressor genes and other genes in tumorogenesis. De novo methylation of CpG sequences in p16, CDH1, MGMT, and APC genes promoter has been described in approximately 30% of colorectal tumors (11-14).

The aim of our study was to determine the prevalence and to analyze the prognostic relevance of p16 promoter methylation using quantitative methylation-specific PCR (qMSP) in a wide cohort of patients with colorectal cancer with long follow-up.

PATIENTS AND METHODS

Sample collection and DNA preparation

The study cohort comprised 326 patients undergoing surgery consecutively for colorectal cancer at the Hospital Clínico San Carlos in Madrid (Spain) between 1995 and 2003. This is a prospective cohort study. All the patients were operated on by the same surgeon who performed radical oncological surgery, based on the location of the tumor. The surgery was defined as curative when there was no evidence of macroscopic residual tumor after resection. Using this criterion, the surgeon performed a curative resection in 269 patients (82.5%) and resected the primary tumor in 57 patients (17.5%) as palliative treatment. Patients with metacronic carcinoma, familial polyposis, familial predisposition for hereditary nonpolyposis colon cancer and inflammatory bowel disease were excluded from the study. None of the patients had received neoadjuvant treatment. Informed consent was obtained from each patient and the project was approved by the clinical research and ethics committee of this hospital. Follow-up was performed according to the protocol designed by the authors (15). Tumors were staged according to Duke's classification. Proximal tumors were defined as occurring in the cecum through to the transverse colon; tumors in the splenic flexure, descending and sigmoid colon were defined as being distal. Stage B and C patients received adjuvant treatment with 5-fluoracil and leucovorin (75% of the patients included). For stage D patients, different protocols were applied according to the Oncology Service criteria.

Tumor and non-tumor tissue samples were obtained during the surgical procedure and immediately immersed in liquid nitrogen for storage in a freezer at -80 ºC. The specimens were then independently examined by two pathologists, who confirmed the samples had more than 80% tumor cells.

For the analysis, DNA was extracted from tumor tissue samples using DNeasy® Blood & Tissue Kit (Qiagen) following the manufacture's instructions.

qMSP

One µg of genomic DNA was subjected to bisulfite modification treatment using and Epitect Bisulfite Kit (Qiagen, Hilden, Germany). The bisulfite-treated DNA was amplified by qMSP, conducted in a Thermal Cycler Real-time System (Smart Cycler, CEPHEID). DNA integrity and modification reaction proficiency were verified using myogenic differentiation 1 (MYOD) gene as internal reference gene. A CpG island free region was selected because its amplification is independent of methylation status. The ratio between Ct value of target gene and Ct value of internal reference gene (MYOD) was used as a measure of the relative methylation level (RML = Ct p16/Ct MYOD), as described previously (16-18). Amplification was carried out in a final volume of 25 ml with QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany) using 1 ml of bisulfite-modified DNA, 10 pmol of each primer and 5 pmol of probes. Primers and probes used in this reaction were:5'-TGGAGTTTTCGGTTGATTGGTT-3'(p16 sense), 5'-TCCTCCACGCCCGCAACAA-3' (p16 antisense), 5'-CGCCAAGCCCCAGCCCA-3'(p16 probe), 5'-GGATTTATATTTATGTGGTGGGTGG-3' (MYOD sense), 5'-TATCTCTCCCCTAAACCTCAACC-3 (MYOD antisense) and 5'-TAGGGGATAGAGGGAGGTGTTTAGGTTG-3' (MYOD probe). Primers and probes chosen for *p16* amplification contained 7 CpG islands and 9 points for bisulfite modification detection (9 cytosines not included in a CpG island). CpG islands discriminate gene methylation status. Negative control (leukocytes from healthy men) and positive control (CpGenome™ Universal Methylated DNA [Chemicon]) were included in each reaction (Fig. 1). The RML cut-off value for this study was fixed at 1.0 for p16 methylation. In thirty patients with proven methylation the non-tumor sample was analysed and none of them had p16 methylation.

Statistical analysis

Qualitative variables are provided with their corresponding frequency distributions. Quantitative variables are expressed as their mean, standard deviation and range. Associations between qualitative variables were evaluated using the χ^2 test or Fisher's exact test when 25% of expected frequencies fell below 5. Overall survival (OS) and disease-free survival (DFS) were estimated by the Kaplan-Meier method and compared among groups using Breslow's exact test. The event in OS was defined as death occurring as a consequence of tumor, censuring live patients and those dying of another cause. OS was calculated as the time elapsed between the date of surgery the date of death or last follow-up. The event in DFS was defined as a diagnosis of locoregional or distant recurrence in patients free from disease. Patients undergoing palliative surgery were excluded for the DFS analysis. The data were fitted to Cox's proportional risks regression model. In each contrast, the null hypothesis was rejected when the type I error was equal or less than 0.05. All statistical tests were performed using SSPS v.11.5 software.

RESULTS

In 326 patients recruited, 53.4% were men and 46.6% women and the median age was 71 years (range 35-95 years). Age was stratified according to median value. Clinicopathological variables are shown in table 1. 62.9% of the tumors were located in colon and 37.1% in rectum. 8.6% of tumors were mucinous adenocarcinomas. In 34 patients differentiation grade could not be assigned.

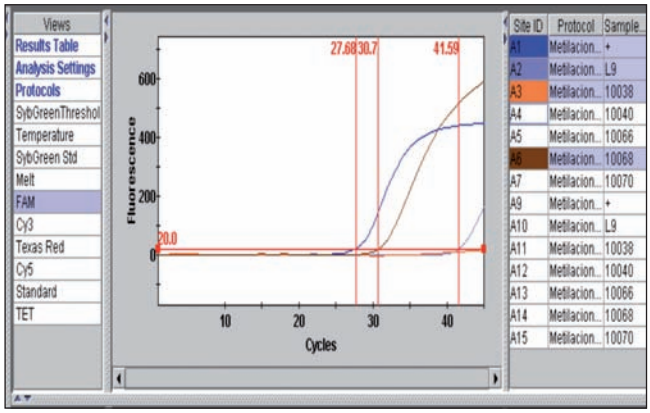

Fig. 1. Melting curve showing positive results for *Positive control* (+: blue) and *Sample 10068* (10068: brown) and negative results for *Negative control: leukocyte* (L9: grey) and *Sample 10038* (10038: orange).

p16 methylation could not be analyzed in 8 patients. Methylation was positive in 24.8% of samples (79 of 318). In the analysis of *p16* and the clinicopathological variables (Table I), we found statistically significant association of methylation and differentiation grade (p = 0.04). In undifferentiated tumors the frequency of methylation was higher (69.2%) than in moderately or well differentiated tumors (24.6 and 21.7%, respectively). Association of *p16* methylation with tumor location was also found (p = 0.02). Methylation was lower in distal colon (16%) than in proximal colon or in rectum (32.4 and 25.9%, respectively). No other significant relation was observed in this analysis.

Table I. Analysis of the relation of *p16* methylation status with the other clinicopathological variables in the 326 colorectal patients. Variables frequencies description

Variable	*n (%)*	*p16 methylate*	*p16 non-methylated*	*p*
Gender	Males: 174 (53.4%) Females: 152 (46.6%)	44 (26.2%) 35 (23.3%)	124 (73.8%) 115 (76.7%)	0.55
Age	≥ 71 years: 170 (52.1%) < 71 years: 156 (47.9%)	48 (29.1%) 31 (20.3%)	117 (70.9%) 122 (79.7 %)	0.06
Dukes	A + B: 174 (53.4%) C: 81 (24.8%) D: 71 (21.8%)	38 (22.1%) 18 (23.4%) 23 (33.3%)	134 (77.9%) 59 (76.6%) 46 (66.7%)	0.12
Tumor location	Proximal: 104 (31.9%) Distal: 101 (31.0%) Rectum: 121 (37.1%)	33 (32.4%) 16 (16.0%) 30 (25.9%)	69 (67.6%) 84 (84.0%) 86 (74.1%)	0.02
Grade *	I: 212 (72.8%) II: 66 (22.7%) III: 13 (4.5%)	45 (21.7%) 16 (24.6%) 9 (69.2%)	162 (78.3%) 49 (75.4%) 4 (30.8%)	0.04
Histological type	Adenocarcinoma: 298 (91.4%) Mucinous: 28 (8.6%)	74 (25.3%) 5 (19.2%)	218 (74.6%) 21 (80.8%)	0.50

*In 35 patients grade could not be established.
The methylation status only was analyzed in 318 patients.

Postoperative course. Overall survival

Median follow-up period was 92 months; with an interquartile range from 75 to 111 months. In our population the OS of our population at this time was 63%. All survival analyses were referred to the median follow-up. During follow-up 142 patients died, 106 of them as a consequence of their neoplasia, and one patient was lost to follow-up. Results of the univariate analysis of OS are shown in table II.

No differences in OS were observed according to *p16* methylation status. OS after 92 months in patients showing *p16* methylation was 62.6 and 64% in the patients without methylation -Hazard ratio-(HR) = 0.91; 95% confidence interval (95% CI = 0.58-1.42). A stratified analysis of OS was performed using the clinicopathological variables. According to this analysis, significant differences were observed in sex (Table III). In males with *p16* methylation the OS was shorter than males without (50.2 *vs.* 63.6%; p = 0,04). We also found a tendency towards negative effect of *p16* in OS of patients with moderately differentiated tumors, OS was 43.8% in those with methylation compared to 59.2% in patients without gene methylation (p = 0.06) (Fig. 2). In multivariable analysis we did not find statistically significant results for *p16* methylation, Dukes stage was the only independent prognostic factor. We performed an exploratory analysis and the corrected HR for the absence of methylation of p16 was 0.88 (95% CI = 0.56-1.39).

Postoperative course. Disease-free survival

DFS in the median follow-up time was 71.6%. Tumor recurrence occurred in 69 patients. The recurrence was locoregional in 14 patients (20%) and distant in 49 patients (80%). Results of the DFS univariate analysis are shown in table II.

In patients with *p16* methylation DFS was 78.2% and in those without this alteration was 70.3% (p = 0.3). Stratified analysis of DFS was performed according to the clinicopathological variables (Table III). Only in differentiation grade a significant effect was proved. Undifferentiated tumors with p16 methylation 0% showed recurrence while in absence of methylation 100% relapsed (p = 0.005) (Fig. 3). In the multivariable analysis for DFS the effect of *p16* methylation was not independent prognostic factor.

Table II. Univariate analysis of OS and DFS referred to the median follow-up time (92 months) in relation with clinicopathologial variables in the 315 colorectal patients studied. Cox analysis

Variables	*Categories*	*OS (%)*	*HR*	*95%CI*	*p*	*DFS (%)*	*HR*	*95%CI*	*p*
Gender	Males	59.5			0.12	70.3	1.2	0.7-1.9	0.45
	Females	67.2	1.3	0.9-1.9		73.0			
Age	≥ 71 years	58.8			0.06	69.9	1.1	0.7-1.8	0.49
	< 71 years	67.7	1.4	0.9-2.0		73.4			
Dukes	A + B	89.0			*p < 0.001*	84.2			*p < 0.001*
	C	60.9	4.2	2.3-7.8		54.1	3.7	2.2-6.3	
	D	6.3	24.7	14.0-43.3		20.5	8.2	3.9-17.2	
Tumor location	Proximal	57.7	1.6	1.0-2.5	0.12	74.9	1.1	0.5-2.1	0.10
	Distal	62.3	1.3	0.8-2.1		62.0	1.7	1.0-3.0	
	Rectum	68.6				77.1			
Grade	I	66			0.08	72.8			0.24
	II	56	1.59	1.0-2.5		73.0	1.0	0.5-1.9	
	III	43	1.86	0.7-4.6		55.6	2.7	0.9-7.6	
Histological type	Adenocarcinoma	65.3	0.57	0.3-1.0	0.07	73.0	0.55	0.2-1.1	0.14
	Mucinous	44.1				54.0			
p16 methylation	Methylated	62.6			0.61	78.2	0.73	0.3-1.3	0.32
	Non- methylated	64.0	0.91	0.58-1.42		70.3			

OS: overall survival; DFS: disease free survival; HR: Hazard ratio; CI: confidence interval. statistical signification: p < 0.05. HR are adjusted in each variable of the table.

DISCUSSION

CRC is the second leading cause of death related to cancer in USA and Europe (19). It is one of the cancers with better characterised tumorogenesis genetic pathways and one of the common alterations implicated in tumorogenesis is DNA methylation (20). Promoter methylation silences tumor suppressor genes in great number of neoplasias (21,22). *p16* gene acts as a negative controller of cell cycle playing an important role as tumor suppressor (23).

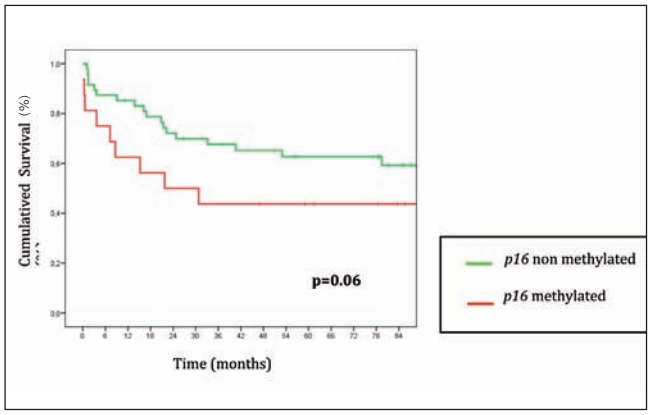

Fig. 2. Kaplan-Meier curves for overall survival in patients with moderately differentiated tumors according to *p16* promoter methylation.

None of the previously published studies of *p16* showed high population size, with so extended follow-up in homogeneous population, only with sporadic cases with no previous treatment (24-29). The only cases who reported higher population size with survival analyses in long follow-up whether had lower median follow up (23) whether consisted of multicenter studies, introducing variability through diverse laboratory analyses and different clinical management, including family history cases (25). In this cohort of 326 patients with sporadic CRC, we evaluated the frequency of *p16* promoter methylation. These frequencies, for CRC patients, have been previously shown to be highly variable, ranging from 18 to 61% (30-36). This variability could be explained by the used of different methodologies in methylation analysis. We previously tested p16 methylation with methylation specific conventional PCR finding 18.3% positive results (33). However, the technique (qMSP) used by the current study increased the positive percentage to 24.8%. qMSP technique can both provide quantitative data, and greater sensitivity; even with small amounts of DNA it is able to detect up to 0.1% methylated alleles in a known CpG island (37).

Methylation of *p16* gene promoter, for CRC patients, is a frequent event. The relation of *p16* methylation with clin-

Table III. Stratified analysis of OS and DFS at the follow-up median in the 318 patients with colorectal cancer

Variable	*p16 methylation*	*OS (92 months)*	*p*	*DFS (92 months)*	*p*
Gender					
Male	Methylated	50.2	*0.04*	69.0	0.71
	Non methylated	63.6		71.2	
Female	Methylated	78.1	0.23	86.0	0.13
	Non methylated	64.3		69.5	
Age					
> 71 years	Methylated	67.4	0.85	79.3	0.29
	Non methylated	56.2		67.0	
< 71 years	Methylated	57.0	0.24	76.2	0.89
	Non methylated	70.5		73.0	
Dukes					
A + B	Methylated	96.9	0.10	88.1	0.42
	Non methylated	86.5		100	
C	Methylated	75.5	0.45	70.6	0.49
	Non methylated	58.3		50.1	
D	Methylated	6.9	0.26	25	0.53
	Non methylated	9.8		25	
Tumor location					
Proximal	Methylated	62.3	0.57	90.5	0.16
	Non methylated	56.4		68.4	
Distal	Methylated	55.6	0.25	54.5	0.43
	Non methylated	64.6		64.1	
Rectum	Methylated	69.6	0.72	79.5	0.79
	No metilado	69.3		77.6	
Histological type					
Adenocarcinoma	Methylated	64.9	0.76	78.8	0.43
	Non methylated	65.9		72.2	
Mucinous	Methylated	40.0	0.22	66.7	0.88
	Non methylated	43.7		47.6	
Grade					
I	Methylated	71.5	0.44	78.4	0.56
	Non methylated	64.8		71.1	
II	Methylated	43.8	0.06	75	0.96
	Non methylated	59.2		71.8	
III	Methylated	57.1	0.37	100	*0.005*
	Non methylated	25.0		0	

OS: overall survival; DFS: disease free survival; Statistical signification: p < 0.05

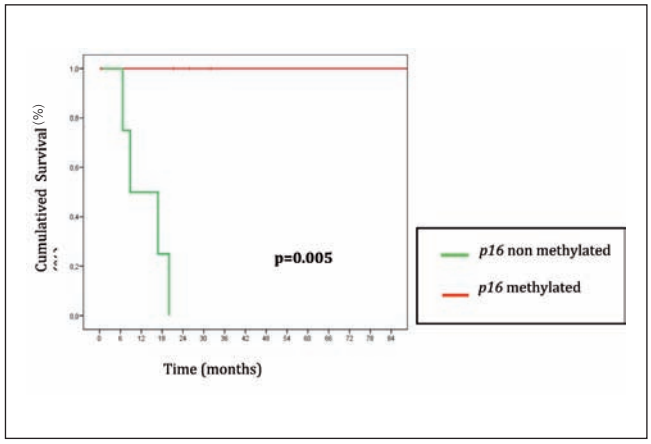

Fig. 3. Kaplan-Meier curves for disease free survival in patients with poorly differentiated tumors according to *p16* promoter methylation.

icopathological variables, in these patients, has yield discrepancies in the literature. Although methylation could not be associated to clinicopathological variables by some authors (28,38), it has been associated to the following: Dukes stage and lymphatic invasion (35); also to age, sex, tumor location, differentiation grade and histological type (36). In this study, the *p16* gene promoter methylation was found to be associated with both, poor differentiation grade and proximal tumor location. Increased frequency of p16 methylation in proximal and poor differentiated tumors has been previously described by Shima y cols. (25). Some authors have described previously described the increased *p16* methylation detected in MSI-H tumors, which are preferentially located in proximal colon (39). MSI status has not been included in our study but could be influencing differences in *p16* tumor location. Relation of *p16* loss of expression, often caused by promoter methylation, and poor differentiation has been described in other malignancies (40,41). Absence of *p16* expression causes deregulation in cell cycle and may lead to rapid cellular division and favours dedifferentiation process.

Overall methylation and also *p16* methylation has been associated with age but this relation only appeared in our cohort as a statistical tendency (25,42). Other authors showed no association of p16 with age, this would explain why none of the normal mucosa analysed showed methylation (43).

Some studies have analysed survival and *p16* promoter methylation (25,26,28,29). In prognosis studies, to work on homogeneous populations and with a long follow-up increases the value of the information obtained (median follow-up 92 months). Although methylation could not be associated to prognosis by some authors, it has been associated with a shorter overall survival (25,28). No significant effect of *p16* methylation in prognosis was observed. However, in the preliminary multivariate analysis, a negative effect on OS was detected, but not demonstrated, due to the population size. Shima y cols. demonstrated in a multivariate analysis the significantly decrease in OS associated with the presence of methylation in *p16* (25).

According to the p16 methylation status, no differences were found in the disease free survival. Some authors have found shorter DFS in the patients showing methylation (29). Unpredictably, in the poor differentiation group (Grade III) we found protective effect of *p16* methylation:

none of the patients showing methylation had recurrence. Other authors had not analysed the effect of p16 in this specific subgroup. The positive effect of *p16* methylation in the prognosis has also been described in gastric tumors (29).

p16 methylation is frequent and induces differences in the DFS of patients with poorly differentiated tumors. However further studies increasing our population size are needed to elucidate p16 effect on the prognosis.

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