p16 Gene Methylation in Colorectal Tumors: Correlation with Clinicopathological Features and Prognostic Value

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Key Words
Methylation · p16 gene · Colorectal cancer · Prognostic value

Abstract

\textbf{Background:} Methylation of the CpG islands in the p16 gene promoter region is an important transcription repression mechanism that has been identified as an alternative mechanism for inactivating specific genes in cancer. Given that, DNA methylation is a common event in colorectal cancer. \textbf{Materials and Methods:} The aim of this study was to establish the methylation status of the p16 gene in 104 patients with colorectal carcinoma and evaluate its prognostic value. DNA was bisulfite-modified and analyzed for p16 promoter methylation by methylation-specific PCR. \textbf{Results:} Methylation of the p16 gene was determined in 18.3\% of our patient population (19/104). The methylated state did not correlate with any clinicopathological factors. During a median follow-up period of 72 months, the overall survival rate for patients with gene methylation was 75\% and without gene methylation it was 61\% (p = 0.09). \textbf{Conclusions:} Although not statistically significant, this difference indicates a clinically valuable tendency.

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Introduction

The p16\textsuperscript{\textsuperscript{INK4a}} gene, also known as CDKN2, MTS1, INK4a and CDK4I, has been identified as a cyclin kinase-dependent inhibitor that can interrupt the tumor cycle by acting as a tumor suppressor gene [1]. Located on chromosome 9p21, this gene is comprised of 3 exons and codes for a 16-kDa protein that inhibits the cyclin kinase D1-CDK4/6 complex responsible for phosphorylating protein Rb. This causes halting of the cell cycle at the G1 stage, at least in cells with functional Rb [2].

Alterations in the 9p21 gene region are common in human cancer [3]. So far, several mechanisms of gene inactivation have been described, including: deletion, methylation of the promoter region, punctual mutations (generally antisense mutations) and open reading frame mutations, their incidence depending on the tumor type [4].

Methylation of the 5’ ends or CG dinucleotide regions called CpG constitutes an important transcription repression mechanism [5]. In normal cells, the CpG islands are only methylated in the inactivated X chromosome. It was reported by Merlo et al. [6] that, despite a loss of heterozygosity in 9p21 being among the most frequent genetic alterations occurring in human cancer, p16 punctual mutations in the other chromosome are relatively uncom-
mon. The methylation of certain genes plays a significant role in tumorigenesis. De novo methylation of CpG sequences in the p16 promoter region has been described in roughly 20% of primary neoplasias.

The aim of our study was to evaluate the prognostic value of methylation of the p16 gene promoter in a cohort of patients with colorectal carcinoma.

**Patients and Methods**

**Patients**

Our prospective cohort study was performed on 104 patients undergoing surgery for primary colorectal carcinoma at the Hospital Clinico San Carlos, Madrid (Spain), between 1995 and 1998. All the patients were operated on by the same surgeon, who performed a radical oncological procedure according to the tumor site. Surgery was defined as curative when there was no macroscopic evidence of residual tumor after resection. According to this criterion, in 82 patients (78.8%) resection was curative and in 22 (21.2%) the primary tumor was resected as palliative treatment. Patients with tumors staged as B, C and D had undergone neoadjuvant treatment. Informed consent to participate was obtained from each patient. The study was approved by our hospital’s clinical research and ethics committee. Patient participation was obtained from each patient. The study was approved by our hospital’s clinical research and ethics committee. Patient follow-up was performed according to a protocol designed by the present authors [7]. Tumor specimens were staged using Dukes’ classification. Patients with tumors staged as B, C and D <80 years of age (75% of the population) were subjected to adjuvant chemotherapy with 5-fluorouracil and leucovorin.

**Method**

**Sample Processing.** A tumor tissue specimen was obtained during the surgical procedure itself and immediately immersed in liquid nitrogen for storage in a freezer at –80°C. The specimens were then independently examined especially for this study by two pathologists, who confirmed the samples had over 80% tumor cells.

**Methylation Status of the p16 Gene Promoter.** Tumor specimens obtained during surgery were treated with proteinase K and RNase. Each specimen was then subjected to DNA extraction using standard phenol-chloroform procedures. We then used 1.0 μg of DNA to establish whether the p16 gene promoter was methylated. To this end, the DNA was denatured with 3.0 M NaOH for 15 min at 37°C and sodium bisulfite-modified at 50°C for 18 h. Sodium bisulfite converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged. Uracil is replicated as thymine during PCR, such that methylation analysis can be undertaken by designing primers that preferentially anneal to sequences containing either methylated (CpG) or unmethylated (TpG) sites. Bisulfite-converted DNA was purified by the Wizard’s resin filter method (Promega). The samples were incubated at 37°C for 15 min in 0.3 M NaOH in 50-μl reaction volumes and repurified on DNA QIAamp columns (Qiagen).

The bisulfite-treated DNA was PCR amplified using primers specific for the methylated CpG and unmethylated regions of the p16 promoter. The PCR reaction mixture contained PCR buffer, MgCl₂ (1.5 mM), deoxynucleotide triphosphate (200 μM each), 0.4 μM of each primer (p16 methylated sense: dTTATAGAGGGTGCGCGGATCGC, p16 methylated antisense: dCCACCTAATCGACCTCGACCG, p16 unmethylated sense: dTATTAGAGGGTGCGGATCGC, p16 unmethylated antisense: dCCACCTAATCGACCTCGACCG), the modified DNA (50 μg) and 2.5 U of Taq polymerase (Roche Diagnostics, Mannheim, Germany) in a final 50-μl volume. The PCR amplification conditions were: 1 cycle at 95°C for 5 min; 35 cycles at 95°C for 30 s, 69°C for 1 min and 72°C for 1 min, and a final extension cycle at 72°C for 10 min.

The resultant PCR products were loaded onto 2% agarose gels and stained with ethidium bromide for subsequent analysis of methylation status under UV light.

**Statistical Analysis**

Qualitative variables are provided with their corresponding frequency distributions. Quantitative variables are expressed as their mean, standard deviation (SE) and range. Associations between qualitative variables were evaluated using the χ² test or Fisher’s exact test when 25% of expected frequencies fell <5. The functions overall survival (OS) and disease-free survival (DFS) were estimated by the Kaplan-Meier method and compared among the groups using Breslow’s exact test. The event in OS was defined as deaths occurring as the consequence of the tumor, ensuring live patients and those dying of another cause. OS was calculated as the time elapsed from the date of surgery until death or last follow-up. The event in DFS was defined as a diagnosis of locoregional or distant recurrence in patients free from disease, i.e., in those in whom curative surgery was performed. The data was fitted to Cox’s proportional risks regression model. In each contrast, the null hypothesis was rejected when the type 1 error was <0.05. All statistical tests were performed using SPSS version 11.5 software.

**Results**

Of the 104 patients enrolled, 51% were men and 49% women. Mean patient age was 69.6 ± 12 years (range 35–91). Table 1 shows the clinicopathological variables of the patients. 52.9% of the tumors were located in the colon and 47.1% in the rectum. 5.8% of the tumors were mucoid adenocarcinomas. In 2 patients, the differentiation grade of the tumor could not be determined.

Methylation of the p16 was detected in 18.3% of the patients (19/104). However, this genetic alteration could not be related to any of the clinicopathological factors analyzed (table 1). It should be noted that none of the patients with a mucoid adenocarcinoma showed gene methylation.

**Postoperative Course: Overall Survival**

The median follow-up period was 72 months (6 years); the interquartile range was from 26 to 104 months.
The OS rate of our study population was 64% at 6 years. All survival analyses were referred to this median follow-up period. During follow-up, 54 patients died; 46 as a consequence of their neoplasia. One patient was lost to follow-up.

The OS of the patients showing p16 methylation was 75% compared to 61% recorded in the patients without the alteration (p = 0.09) (fig. 1). In an analysis of OS performed by stratifying according to the classic prognostic factors used to predict the risk of death in patients with colorectal cancer, only the degree of tumor differentiation showed a significant effect. Thus, among the patients with well-differentiated tumors (grade I), OS was 93% for those with p16 methylation compared to 68% for patients without gene methylation (p = 0.05). Our multivariate analysis revealed that the p16 alteration had no prognostic value independent of the remaining variables for predicting the risk of death.

**Postoperative Course: Disease-Free Survival**

The DFS rate for the study population was 75%. Tumor recurrence occurred in 25 patients during follow-up. The site of recurrence was locoregional in 5 patients (20%) and distant in 20 patients (80%).

**Table 1.** Clinicopathological characteristics of 104 patients with colorectal carcinoma according to the methylation status of the p16 promoter

<table>
<thead>
<tr>
<th>Variable</th>
<th>Methylated p16</th>
<th>Unmethylated p16</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 70 years</td>
<td>11 (17.7)</td>
<td>51 (82.3)</td>
<td>0.8</td>
</tr>
<tr>
<td>&lt;70 years</td>
<td>8 (19.0)</td>
<td>34 (81.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men: 53 (51)</td>
<td>10 (18.9)</td>
<td>43 (81.1)</td>
<td>0.8</td>
</tr>
<tr>
<td>Women: 51 (49)</td>
<td>9 (17.6)</td>
<td>42 (82.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Dukes’ stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A+B: 48 (46.2)</td>
<td>11 (22.9)</td>
<td>37 (77.1)</td>
<td>0.4</td>
</tr>
<tr>
<td>C: 26 (25)</td>
<td>3 (11.5)</td>
<td>23 (88.5)</td>
<td></td>
</tr>
<tr>
<td>D: 30 (28.8)</td>
<td>5 (16.7)</td>
<td>25 (83.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon: 55 (52.9)</td>
<td>11 (20)</td>
<td>44 (80)</td>
<td>0.6</td>
</tr>
<tr>
<td>Rectum: 49 (47.1)</td>
<td>8 (16.3)</td>
<td>41 (83.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I: 80 (78.4)</td>
<td>16 (20)</td>
<td>64 (80)</td>
<td>0.4</td>
</tr>
<tr>
<td>II-III: 22 (21.6)</td>
<td>3 (13.6)</td>
<td>19 (86.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Adenocarcinoma: 98 (94.2)</td>
<td>19 (19.4)</td>
<td>79 (80.6)</td>
<td>0.1</td>
</tr>
<tr>
<td>Mucoid: 6 (5.8)</td>
<td>0 (0)</td>
<td>6 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Percentage values are shown in parentheses.

<sup>1</sup> Mean patient age.

Fig. 1. OS rates of patients with colorectal cancer according to p16 promoter methylation status.
Patients with p16 methylation showed a DFS of 75% and those without this alteration one of 65% (p = 0.3). An analysis of DFS was performed by stratifying according to the classic prognostic factors able to predict the risk of recurrence in patients with colorectal carcinoma. None of the factors analyzed proved to be significant.

Discussion

In early studies, gene hypermethylation status was analyzed through Southern blotting, a technique that shows poor specificity and requires large amounts of DNA. In 1996, Herman et al. [8] developed a simpler, more specific method that requires a smaller DNA sample. This technique is based on altering the DNA by treatment with sodium bisulfite, and subsequent amplification of methylated and unmethylated sequences rich in CpG islands. The prevalence of p16 hypermethylation in colorectal tumors reported in the different studies varies according to the technique used so we will center our discussion on works based on the Herman technique. These studies report a percentage of hypermethylation ranging between 17 and 42% [9–14]. In the present study, the proportion of patients showing p16 promoter methylation was 18.3% (19 out of 104 patients). This genetic aberration was found not to be related to any of the clinicopathological factors examined. This issue has yielded discrepancies in the literature. Thus, Liang et al. [13] were also unable to correlate hypermethylation with clinicopathological variables, while Yi et al. [14] observed an association with more advanced Dukes’ stages (C and D) (p = 0.01); Shannon and Iacopetta [9], Wiencke et al. [11] and Burri et al. [12] with undifferentiated tumors and those proximally located, and Wiencke et al. [11] with advanced age and female gender.

Investigation into the genetic alterations associated with the development of a colorectal tumor has yielded two hypotheses: one whereby the tumor arises from chromosome instability, implying a sequential change in oncogenes and suppressor genes, and the other involving a microsatellite instability (MSI) or mutant phenotype resulting from an alteration in the repair genes, particularly hMLH1 and hMSH2 [15–18]. MSI is characterized by alterations in nucleotide repeat sequences, denoted microsatellites, and this mechanism is associated with a better prognosis. In 1997, Ahuja et al. [19] reported a relationship between MSI and hypermethylation of the p16 promoter. This relationship may be explained by the theory that epigenetic changes promote the methylation of some gene promoters including those of the p16 and hMLH1 genes leading to blockage of their transcription. These changes involve the alteration of the repair genes conditioning the MSI phenotype [20]. It is generally accepted that patients who develop tumors through the MSI mechanism show an improved prognosis [21, 22]. However, Liang et al. [13], despite noting an association between p16 methylation and the mutator phenotype, observed a worse progression in patients with methylated p16. They proposed that geographical differences or other unknown factors added to this alteration might increase tumor aggressiveness. There are no other reports in the literature evaluating the prognostic role of p16 hypermethylation.

In our patients, those with p16 methylation showed a 6-year OS of 75% compared to the 64% recorded for patients without the alteration. Though not significant (p = 0.09), this difference suggests a clear tendency. This tendency was especially noted in the well-differentiated tumors for which OS was 93% in patients showing hypermethylation and 68% in those not showing the alteration (p = 0.05). It would therefore be interesting to establish the mutator phenotype in this patient population, to confirm its association with the p16 alteration and its possible prognostic value.

Acknowledgement

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References