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High frequency of *MLH1* promoter methylation mediated by gender and age in colorectal tumors from Mexican patients

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Abstract

Introduction: *Several genes determine the development of colorectal cancer (CRC), such as MLH1, which encodes a protein that participates in DNA repair. MLH1 hypermethylation has been associated with gene silencing.* **Objective:** *To analyze the methylation of five regions of MLH1 CpG island in colorectal tumors from Mexican patients.* **Materials and methods:** *One hundred and one tumor tissue samples were obtained from Mexican patients with CRC who provided informed consent. DNA was subjected to bisulfite conversion. Methylation of all five regions of the CpG island was evaluated using methylation-specific PCR.* **Results:** *The frequency of methylation in Mexican patients with CRC was 25%. Regions A and B methylation was the main observed pattern (60%). Female patients showed a higher frequency of methylation (71%; OR: 3.085; CI: 1.85-8.03; p = 0.02), and out of total methylated samples, 80% corresponded to individuals older than 45 years (p < 0.05).* **Conclusion:** *We calculated a methylation frequency for the MLH1 gene of 25% in Mexican patients with CRC, with this being the first report for this population. Female patients and patients older than 45 years showed a higher frequency of methylation.*

KEY WORDS: *Colorectal cancer. Methylation Mexican population. MLH1.*

Elevada frecuencia de metilación del promotor de MLH1 mediada por sexo y edad en tumores colorrectales de pacientes mexicanos

Resumen

Introducción: *Varios genes determinan el desarrollo de cáncer colorrectal (CCR), como MLH1, el cual codifica una proteína que participa en la reparación del ADN. La hipermetilación de MLH1 ha sido asociado con silenciamiento génico.* **Objetivo:** *Analizar la metilación de cinco regiones de la isla CpG de MLH1 en tumores colorrectales de pacientes mexicanos.* **Materiales y métodos:** *Se obtuvieron 101 muestras de tejido tumoral de pacientes mexicanos con CCR, quienes proporcionaron su consentimiento informado. El ADN fue sometido a conversión por bisulfito. La metilación de las cinco regiones de la isla CpG fue evaluada utilizando PCR específica para metilación.* **Resultados:** *La frecuencia de metilación en pacientes mexicanos con CCR fue del 25%. La metilación de las regiones A y B fue el principal patrón observado (60%). Las pacientes de sexo femenino mostraron una mayor frecuencia de metilación (71%) (odds ratio: 3.085; intervalo de confianza; 1.85-8.03;*

p = 0.02); y del total de muestras metiladas, el 80% fueron individuos mayores de 45 años (p < 0.05). **Conclusión:** *Calculamos una frecuencia de metilación para el gen MLH1 del 25% en pacientes mexicanos con CCR, siendo el primer reporte para esta población. Pacientes de sexo femenino y pacientes mayores de 45 años mostraron una mayor frecuencia de metilación.*

PALABRAS CLAVE: Cáncer colorrectal. Metilación. Población mexicana. MLH1.

Introduction

Colorectal cancer (CRC) is the third leading cause of tumor-related death in the world[1,2]. Approximately 45% of cases occur in developing regions[1,3]. CRC usually occurs in adults with an average age of 67 years[4]. In Mexico, in terms of incidence and mortality, CRC is the third most common cancer in both genders[1].

CRC is a neoplasm in which epigenetic processes such as CpG islands hypermethylation occur, which leads to gene inactivation and normal cell growth and function alteration[5]. *MLH1* hypermethylation has been described as a prognostic marker for this neoplasm[6]. The MLH1 protein is part of a protein complex that participates in the mismatch repair system[7-9]. The *MLH1* gene is located at 3p22.2 and contains 19 exons[10]. Its expression is regulated by its promoter, and although it lacks a TATA box, it contains the CCAAT sequence that is necessary for transcription factors binding[11]. *MLH1* has a 1128-bp CpG island, where 93 CpG sites are found (chr3: 37034229–37035356)[12]. In sporadic CRC, *MLH1* is methylated at 10-15%[5]. Four regions of the CpG island have been studied in colorectal carcinoma cell lines: A (-711 to -577), B (-522 to -266), C (-248 to -178) and D (-109 to +5), with hypermethylation being correlated with *MLH1* gene expression inhibition[13,14]. On the other hand, in the Japanese population, analysis of five regions has been reported: A (-755 to -574), B (-597 to -393), C (-420 to -188), D (-286 to -53) and E (-73 to +86), with total and partial methylation being correlated with clinical-pathological characteristics, protein expression and microsatellite instability[15].

There are no specific *MLH1* methylation reports in Mexican patients with CRC. Owing to this, the purpose of the present study was to analyze the methylation frequency of five regions of the *MLH1* CpG island in Mexican patients with CRC.

Materials and methods

Samples

One hundred and one tumor tissue samples were collected from Mexican patients clinically and histopathologically diagnosed with CRC at Dr. Juan I. Menchaca Civil Hospital (Guadalajara, Jalisco, Mexico). Tissue samples were obtained at the conclusion of surgery. All patients signed an informed consent based on the Declaration of Helsinki. The study was approved by the local bioethics committee (CI-01417).

DNA extraction and quantification

DNA was extracted from tumor tissues (High Pure PCR Template Preparation kit [product no.: 11796828001], Roche Diagnostic GmbH, Mannheim, Germany). Subsequently, the DNA was quantified by spectrophotometry. Samples were stored at -20 °C until their use.

DNA conversion by bisulfite

A DNA concentration of 100 μg/mL (18 μL) was used and subsequently treated with the EZ DNA Methylation-Gold kit (product no.: D5006; ZYMO Research, USA). DNA from the HCT116 DKO cell line (product no.: D5014; ZYMO Research) was used as control during the conversion analysis and methylation-specific polymerase chain reaction (MS-PCR).

Methylation-specific polymerase chain reaction

The CpG island was divided into five regions for methylation analysis: A (-539 to -677), B (-418 to -264), C (-162 to -62), D (+82 to +260) and E (+234 to +415). MS-PCR was carried out for all regions using converted DNA. The primers used for methylated and unmethylated DNA are described in table 1. MS-PCR was carried out for all regions at 30 cycles, in a volume of 12 μL, which contained 100 ng/μL DNA, 1 x buffer (500 mM KCl, 100 mM Tris-HCl and 0.1% Triton™ X-100), 1.5 mM MgCl$_2$, 2 mM dNTP, 10 pM of each primer and 2 U of Platinum Taq DNA polymerase. Initial denaturation was carried out at 95 °C for 5 min, followed by 94 °C for 45 s, alignment at 57 °C for 45 s and elongation at 72 °C for 1 min. The PCR products

Table 1. *MLH1* gene primers used for A, B, C, D, and E regions methylated and unmethylated DNA amplification

CpG island regions	M/U	Primers	Fragment size (bp)
A	M	F5'-CGGTAGAGTTCGAGGTTTGTAC-3'	134
		R5'-CACGAATACTACGAACGATATATAACG-3'	
	U	F5' GTGGTAGAGTTTGAGGTTTGTATGA-3'	138
		R5'-AAACACAAATACTACAAACAATATATAACA-3'	
B	M	F5'- GTCGGAAAATTAGAGTTTCGTC-3'	151
		R5'-GCAAAACGAAAAAAATACTTAACG-3'	
	U	F5'- GGTTGGAAAATTAGAGTTTTGTTGA-3'	154
		R5'-ACAAAACAAAAAAAATACTTAACACA-3'	
C	M	F5'-GATAGCGATTTTTAACGC-3'	93
		R5'-TCTATAAATTACTAAATCTCTTCG-3'	
	U	F5'-AGAGTGGATAGTGATTTTTAATGT-3'	100
		R5'-ACTCTATAAATTACTAAATCTCTTCA-3'	
D	M	F5'-GTTTTTTTGGCGTTAAAATGTC-3'	166
		R5'- CCTTAAATAAACCCGACTCGAC-3'	
	U	F5' -TTGGTTTTTTTGGTGTTAAAATGTT-3'	172
		R5'-AACCCTTAAATAAACCCAACTCAAC-3'	
E	M	F5'-GAGTCGGGTTTATTTAAGGGGTTAC-3'	177
		R5'-GATAAAAAAACACACGATCTACGAA-3'	
	U	F5'-AGTTGGGTTTATTTAAGGGGTTATGA-3'	176
		R5'- AATAAAAAAACACACAATCTACAAA-3'	

M: methylated; U: unmethylated; bp: base pairs; F: forward; R: reverse.

were visualized on polyacrylamide gels stained with 6% silver nitrate.

Statistical analysis

SPSS v25 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Fisher's exact test, chi-square test and odds ratios (OR), were used for methylation analysis. p-values < 0.05 were regarded as significant.

Results

One hundred and one tumor tissue samples from Mexican patients with CRC were analyzed; average age was 59 years. Analyzed characteristics included gender (52% males), physical activity (53%), type II diabetes mellitus (21%), consumption of tobacco, alcohol, red meat and fruits and vegetables (42, 49, 81 and 75%, respectively), metastasis (42%), presence of polyps (17%), relapses (4%) and tumor location (colon, 60%).

Methylation analysis

The frequency of *MLH1* methylation in Mexican patients with CRC was 25%. However, among total methylated samples, methylation frequency was 60% in regions A and B (Table 2).

Methylation frequency was compared according to clinical-pathological and lifestyle characteristics, with significant differences being found for gender and age (p < 0.05) (Table 3). Of total methylated samples, 68% corresponded to female patients. In addition, two age ranges were established (< 45 and > 45 years of age); out of total methylated samples, 80% corresponded to patients older than 45 years.

Table 2. Methylation analysis of *MLH1* gene CpG island regions of tumor tissue from patients with colorectal cancer

Methylated region	Total methylated samples
	n = 25 (%)
A	15 (60)
B	15 (60)
C	7 (28)
D	8 (32)
E	7 (28)

The frequency of methylation (25%) was compared with the frequencies reported in other populations, with significant differences being found (Table 4).

Discussion

Methylation analysis

In the present study, five CpG island regions were analyzed. It should be noted that regions D and E had not been previously analyzed by other authors (Table 5). The five analyzed regions cover a total of 63 CpG island sites.

Previous studies analyzed four regions rich in CpG sites by means of sequencing; however, cell lines were used, covering 43 CpG sites. In particular, these studies showed a correlation between C or D region methylation and gene expression inhibition due to the presence of the CCAAT sequence; methylation of this sequence inhibits transcription core binding factor (CBF) binding. According to these studies, hypermethylation in *MLH1* promoter C and D regions causes inactivation and deficiency of the protein[13,14]. In our study, 28% of the samples were found to be methylated at C region and 32% were methylated at D region. These regions are found around the transcription start site, but always have a lower methylation frequency than the other regions[22]. For example, regions A and B are the most commonly methylated[23]; however, the functional effect of these regions has not been directly described. In this regard, in the present study, a higher frequency of methylation was observed in regions A and B (60%). Another report revealed that region A may be the first site to be methylated during progression to a methylation state[15]. These regions (A, B) are the furthest from the transcription start site. On the other hand, all methylated tumors in region C showed methylation in regions A and B, which was a constant in our study, but the authors showed cases of methylation only in regions A and B, and not in region C[13].

Methylation frequency was 25% for *MLH1*. DNA methylation is known to be able to affect transcription factor binding sites, regulatory elements and chromatin conformation, which results in multiple levels of expression control[24]. This frequency is higher than that proposed by other authors, who refer a *MLH1* methylation frequency of between 10 and 15% in cases of sporadic CRC[5]. Even our percentage was also higher than that reported in other populations[7,16-21].

Gender-mediated methylation

CRC is a complex disease that involves several factors[25-28]. In the present study, the correlation of some of these factors with methylation was examined. However, there were only significant differences with regard to gender and age. Specifically, *MLH1* methylation has been associated with clinical-pathological variables, including the female gender[29]. In this study, the frequency of methylation was higher in women (OR: 3.085; confidence interval [CI]: 1.85-8.03; p = 0.02); these findings are similar to those reported in different studies[16,18]. A report that included 210 samples from patients with CRC revealed higher methylation in women (p = 0.007). In addition, the presence of the *MLH1* c.-93G>A variant (rs1800734) was proposed to lead to a higher risk of methylation in women[21]. Interestingly, in this work, those women who had some methylated region had the presence of overweight or obesity in common, as well as that at some stage of their life they had consumed tobacco and alcohol. In this regard, there is sufficient evidence of the influence of these factors on DNA methylation[30-32].

Age-mediated methylation

The methylation frequency analysis by age range revealed higher rates of methylation in individuals older than 45 years (80%). However, the effect of age on methylation status remains unclear. The only study involving *MLH1* that associated aging with aberrant methylation showed that methylation increased with age (frequency per 10 years of age = 2.1%; p < 0.001)[33]. Furthermore, *MLH1* methylation in tumor samples was found to occur more frequently in those from subjects

Table 3. Comparison of *MLH1* gene CpG island regions methylation frequency by gender

Regiones	CRC patients (n = 101)				p	OR (CI)
	Male gender (n = 53)		Female gender (n = 48)			
	M	U	M	U		
Any region*	8 (15.1%)	45 (84.9%)	17 (35.4%)	31 (64.6%)	0.018	3.085 (1.9-8.0)
A	3 (5.7%)	50 (94.3%)	12 (25.0%)	36 (75.0%)	0.01	5.556 (1.5-21.1)
B	4 (7.5%)	49 (92.5%)	11 (22.9%)	37 (77.1%)	0.048	3.642 (1.1-12.4)
A+B	2 (3.8%)	51 (96.2%)	9 (18.8%)	39 (81.3%)	0.023	5.885 (1.2-28.8)

*Samples methylated on any analyzed region (A, B, C, D, E).
CRC: colorectal cancer; M: methylated; OR: odds ratio; CI: confidence interval; U: unmethylated.

Table 4. *MLH1* gene methylation frequency between different populations of patients with colorectal cancer

Population	n	Analyzed tumor tissue	M (%)	p	Reference
Australian	946	Fresh tissue	10.1	0.0001	Wong et al., 2011[16]
Japanese	104	Fresh tissue	9.6	0.0039	Hokazono et al., 2014[17]
Japanese	210	Fresh tissue	28.5	0.4935	Miyakura et al., 2014[18]
Chinese	301	Fresh tissue	14.6	0.0188	Wang et al., 2014[19]
Spanish	326	Fresh tissue	5.2	0.0001	Veganzones et al., 2015[20]
Korean	132	Fresh tissue	6	0.0001	Lee et al., 2019[21]
Slovakian	300	Paraffin-embedded tissue	7.6	0.0001	Kašubová et al., 2019[7]
Mexican	101	Fresh tissue	25	-	Present study

M: methylated alleles percentage.

Tale 5. Comparison between different *MLH1* gene CpG island regions reported by Deng et al. (2001), Miyakura et al. (2001 and 2014) and this study

Region	Deng et al., 2001[14]	Miyakura et al., 2001[15] Miyakura et al., 2014[18]	This study
A	–711 to –577	–755 to –574	–677 to –539
B	–552 to –266	–597 to –393	–418 to –264
C	–248 to –178	–420 to –188	–162 to –62
D	–109 to +5	–286 to –53	+82 to +260
E		–73 to +86	+234 to +415

older than 50 years[29]. In our study, elevated levels of methylation were more common in women older than 45 years.

Population methylation analysis

The population analysis showed that the frequency of methylation in patients with CRC in the Mexican population was significantly different from those in other populations, including Australian, Japanese, Chinese, Spanish, Korean and Slovakian populations[7,16-21]. These differences are probably due to the variability in the number of samples used at each study, the different regions analyzed by each author, the lifestyle and characteristic genetic structure of Mexicans. However, in this study, no significant data were found when the association of methylation with aspects related to lifestyle was analyzed.

Conclusion

The present analysis showed an *MLH1* methylation frequency of 25%. This value is higher than that reported in other populations and constitutes the first report in Mexican patients with CRC. Analysis by regions revealed that regions A and B had a higher methylation frequency (60%). Finally, 80% of methylated samples corresponded to patients older than 45 years, and high levels of methylation were found in female patients, which indicates a relevant role of both these factors in DNA methylation.

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Conflict of interests

The authors declare that they have no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures that were followed adhered to the standards of the research ethics committee and to the World Medical Association code of ethics (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained written informed consent from the patients or subjects mentioned in the article. This document is in the possession of the corresponding author.

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