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RESEARCH ARTICLE

Reduced mRNA expression levels of MBD2 and MBD3 in gastric carcinogenesis

Thaís Brilhante Pontes · Elizabeth Suchi Chen · Carolina Oliveira Gigek · Danielle Queiroz Calcagno · Fernanda Wisnieski · Mariana Ferreira Leal · Samia Demachki · Paulo Pimentel Assumpção · Ricardo Artigiani · Laércio Gomes Lourenço · Rommel Rodriguez Burbano · Marília Arruda Cardoso Smith

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Abstract Aberrant methylation has been reported in several neoplasias, including gastric cancer. The methyl-CpG-binding domain (MBD) family proteins have been implicated in the chromatin remodeling process, leading to the modulation of gene expression. To evaluate the role of *MBD2* and *MBD3* in gastric carcinogenesis and the possible association with clinicopathological characteristics, we assessed the mRNA levels and promoter methylation patterns in gastric tissues. In this study, *MBD2* and *MBD3* mRNA levels were determined by RT-qPCR in 28 neoplastic and adjacent nonneoplastic and 27 gastritis and non-gastritis samples. The promoter methylation status was determined by bisulfite sequencing, and we found reduced *MBD2* and *MBD3* levels in the neoplastic samples compared with the other groups. Moreover, a strong correlation between the *MBD2* and *MBD3* expression levels was observed in each set of paired samples. Our data also showed that the neoplastic tissues exhibited higher *MBD2* promoter methylation than the other groups. Interestingly, the non-gastritis group was the only one with positive methylation in the *MBD3* promoter region. Furthermore, a weak correlation between gene expression and methylation was observed. Therefore, our data suggest that DNA methylation plays a minor role in the regulation of *MBD2* and *MBD3* expression, and the presence of methylation at CpGs that interact with transcription factor complexes might also be involved in the modulation of these genes. Moreover, reduced mRNA expression of *MBD2* and *MBD3* is implicated in gastric carcinogenesis, and thus, further investigations about these genes should be conducted for a better understanding of the role of abnormal methylation involved in this neoplasia.

Keywords Methyl-binding domain proteins · Gastric cancer · DNA methylation · Gene expression

T. B. Pontes · E. S. Chen · C. O. Gigek · D. Q. Calcagno · F. Wisnieski · M. F. Leal · M. Arruda Cardoso Smith (✉)
Disciplina de Genética, Departamento de Morfologia e Genética, Universidade Federal de São Paulo, Rua Botucatu, 740 – Ed. Leitão da Cunha – 1ºandar, São Paulo 04023-900, SP, Brazil
e-mail: macsmith.morf@epm.br

S. Demachki
Serviço de Patologia, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, Belém, PA, Brazil

P. P. Assumpção
Serviço de Cirurgia, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, Belém, PA, Brazil

R. Artigiani
Departamento de Patologia, Universidade Federal de São Paulo, São Paulo, SP, Brazil

L. G. Lourenço
Disciplina de Gastroenterologia Cirúrgica, Departamento de Cirurgia, Universidade Federal de São Paulo, São Paulo, SP, Brazil

R. R. Burbano
Laboratório de Citogenética Humana, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil

Introduction

Gastric cancer, the fourth most common cancer and the second leading cause of cancer death worldwide, is a major public health problem, even though its incidence and mortality are now gradually decreasing [1]. It is known that the development and progression of cancer is a consequence of progressive accumulation of different genetic and epigenetic alterations. Moreover, epigenetic modifications, especially DNA methylation, have been shown to have a central role in gastric carcinogenesis [2–4].

Chromatin remodeling, which includes DNA methylation and histone posttranslational modifications, can modulate DNA function, regulating chromatin structure and determining its transcriptional state [5]. The crosstalk between DNA methylation and histone modifications is established by different nuclear factors, such as methyl-CpG-binding domain protein (MBD). MBD family proteins are associated with various chromatin modifiers to establish a repressive chromatin environment. *MBD2* and *MBD3* have a long homologous sequence, and their proteins are essential subunits of the nucleosome remodeling and deacetylase (NuRD) complex [6, 7] involved in chromatin remodeling [8]. The NuRD complex has been shown to bind to aberrantly methylated promoter regions in several types of cancer [9] and has a dual role in both promoting and suppressing tumorigenesis through several processes, such as transcription, chromatin assembly, cell cycle progression, and genomic stability [10].

Thus, the generation of novel aberrantly methylated regions during cancer development and progression makes MBD proteins interesting targets due to their biological and clinical implications. Several studies on abnormal patterns of methylation in cancer have been conducted; however, the knowledge regarding the impact of these changes in the gastric cancer methylation machinery is still limited. Therefore, we aimed to investigate the impact of *MBD2* and *MBD3* gene expression levels in gastric cancer and the possible association between these findings and clinicopathological characteristics. Moreover, we also determined whether DNA methylation was responsible for the gene expression alterations.

In the present study, we describe for the first time (a) *MBD3* mRNA expression measurements in gastric cancer, (b) *MBD2* and *MBD3* mRNA expression correlations, and (c) *MBD2* and *MBD3* mRNA expression and methylation patterns in samples with different histopathology (neoplastic, adjacent nonneoplastic, gastritis, and non-gastritis samples). These findings allow the analysis of possible preneoplastic changes that may result in a better understanding of gastric cancer progression.

Materials and methods

Clinical samples

Gastric tissue samples ($n=28$) were obtained from patients with gastric cancer who underwent surgical treatment during the years 2009 to 2011 and agreed being part of the research at the *Hospital Universitário João de Barros Barreto* and *Hospital São Paulo*, Brazil. Neoplastic (N) and paired adjacent nonneoplastic (ANN) specimens were immediately cut from the resected stomach. Subjects were not exposed to either chemotherapy or radiotherapy before surgery. We also collected gastritis (G) and paired non-gastritis tissues (NG) ($n=27$) from subjects who underwent gastric endoscopy at the Hospital Universitário João de Barros Barreto. All samples were frozen in liquid nitrogen and kept at −80 °C for later RNA and DNA extraction. The gastric cancer samples were classified according to Laurén [11].

The Institutional Research Ethics Committee approved this study, and all participants or their representatives were informed about the study protocol and provided informed consent according to the Declaration of Helsinki.

Gene expression

RNA was extracted using an Allprep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA concentration and quality were determined with a NanoDrop spectrophotometer (Kisker, Steinfurt, Germany) and 1 % agarose gel. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol.

The expression levels of *MBD2* (Hs00187506_m1) and *MBD3* (Hs00172710_m1) (Applied Biosystems, Foster City, CA, USA) were evaluated in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as well as the most stable internal reference genes, *B2M* (beta-2-microglobulin) (Hs00984230_m1) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (Hs99999905_m1), which were previously evaluated [12]. All samples were run in triplicate, and a non-template control was used in all of the plates. The gene expression values were calculated using the ΔΔCt method, as previously described [13].

DNA methylation analysis

To assess the methylation status of the CpG islands in the promoter regions of the *MBD2* and *MBD3* genes, the DNA samples were treated with an EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany), and the primers (*MBD2*: F–GTTTAGGA GGGAATTGGTATT and R–TAATCRAACTAACCACCA ACTA; *MBD3*: F–AAAAAAAGTTATTGGAGGGAAT and R–ACCAATACCCAACAACTATACC) were designed using Methyl Primer Express® Software v1.0 (Applied Biosystems, Foster City, CA, USA). The bisulfite-treated DNA samples were amplified, purified, and sequenced using an ABI3500 (Applied Biosystems, Foster City, CA, USA). The sequencing results were aligned and analyzed using *CpG Viewer* 1.0 software [14]. The methylation status of the promoter regions was calculated as the frequency of methylated CpGs within the analyzed region.

To determine which transcription factors were predicted to bind to the analyzed CpG islands, we used the online tool PROMO 3.0 [15, 16].

Statistical analysis

The mRNA levels of *MBD2* and *MBD3* among the groups and the clinicopathological features were analyzed by non-parametric tests, including the Mann–Whitney or Kruskal–Wallis tests. A Spearman's correlation coefficient was performed to assess the relationship between the mRNA expression levels of both genes as well as the mRNA and methylation patterns. All statistical analyses were carried out in SPSS 20.0 (SPSS, Chicago, IL, USA). The data are shown as the median and interquartile range, and p values lower than 0.05 were considered significant. Multiple comparison corrections were used when applicable.

Results

We first asked whether genes involved in the methylation machinery, such as *MBD2* and *MBD3*, had abnormal levels of expression in gastric tissues at different histopathological stages. *MBD2* mRNA expression was decreased in the N group compared with the ANN ($p=0.0127$), G ($p=0.0001$), and NG ($p=0.0001$) groups. Moreover, the *MBD2* mRNA expression of the ANN group was reduced in comparison with the G ($p=0.015$) and NG ($p=0.032$) groups (Fig. 1a). When the *MBD3* mRNA expression was analyzed, significant differences between the N and ANN groups ($p=0.047$) and between the ANN and G groups ($p=0.015$) were found (Fig. 1b). However, only *MBD2* expression levels of the N group remained significantly different from the G and NG ($p<0.0001$) groups after multiple comparison corrections (Fig. 1a).

We also observed a strong correlation between the mRNA expression levels of *MBD2* and *MBD3* in both neoplastic and nonneoplastic tissues ($r=0.783$; $p<0.001$ and $r=0.679$; $p<0.001$, respectively; Fig. 2a) and between non-gastritis tissue samples ($r=0.600$; $p=0.018$; Fig. 2b).

We also evaluated the association between the *MBD2* and *MBD3* mRNA expression levels and clinicopathological features, but no significant associations were found (Table 1).

We next asked whether the differences in the gene expression levels found in our samples were due to abnormalities in the methylation status of the promoter regions of *MBD2* and *MBD3*. The gastric neoplastic tissue (N) showed a higher *MBD2* promoter methylation level (17.8 %) than the ANN (10.71 %), G (4 %), and NG (8 %) groups as shown in Table 2. Interestingly, our findings showed no methylation in the promoter region of *MBD3* only in the NG group (19.4 %) and not in the G, ANN, and N groups. When a paired analysis was performed, the *MBD2* and *MBD3* methylation status was not different between the N and ANN or between the G and NG tissues ($p>0.05$).

After the methylation analysis, we examined whether there was a correlation between the mRNA expression and DNA methylation in the promoter regions of these genes. The *MBD2* expression showed a poor inverse correlation with the promoter methylation pattern in the N ($r=-0.098$), ANN ($r=-0.056$), and G ($r=-0.099$) group samples. For the *MBD3* gene, this inverse correlation was observed only in the NG group ($r=-0.216$).

Discussion

In cancer, the role of MBD proteins has been associated with their function as transcriptional repressors or chromatin remodelers [17–19] involved in the silencing of methylated genes [8]. MBD proteins have been associated with the

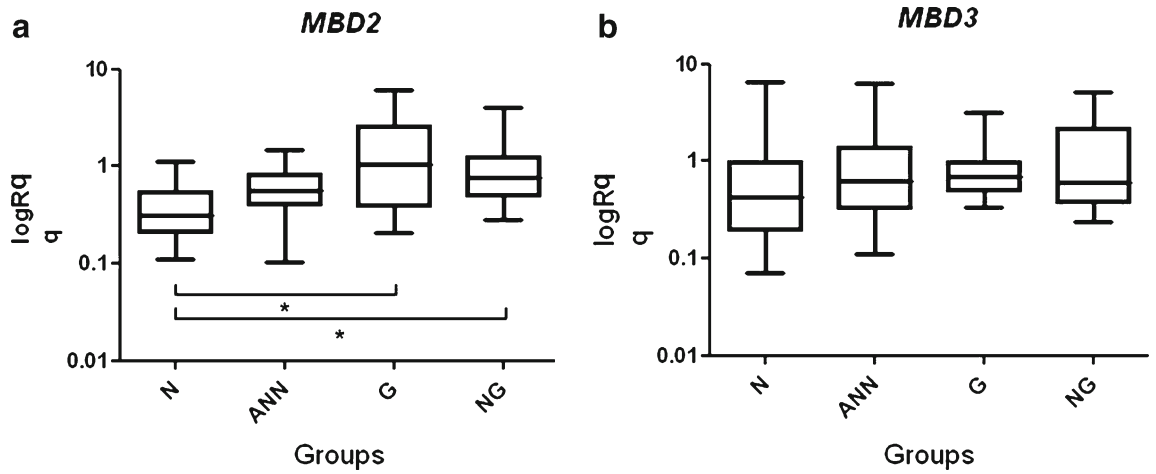

Fig. 1 Relative quantification (logRq) of *MBD2* (**a**) and *MBD3*; **b** mRNA expression in the tumor (N), paired non-tumor (ANN), gastritis (G), and paired non-gastritis (NG) groups. *$p<0.0001$

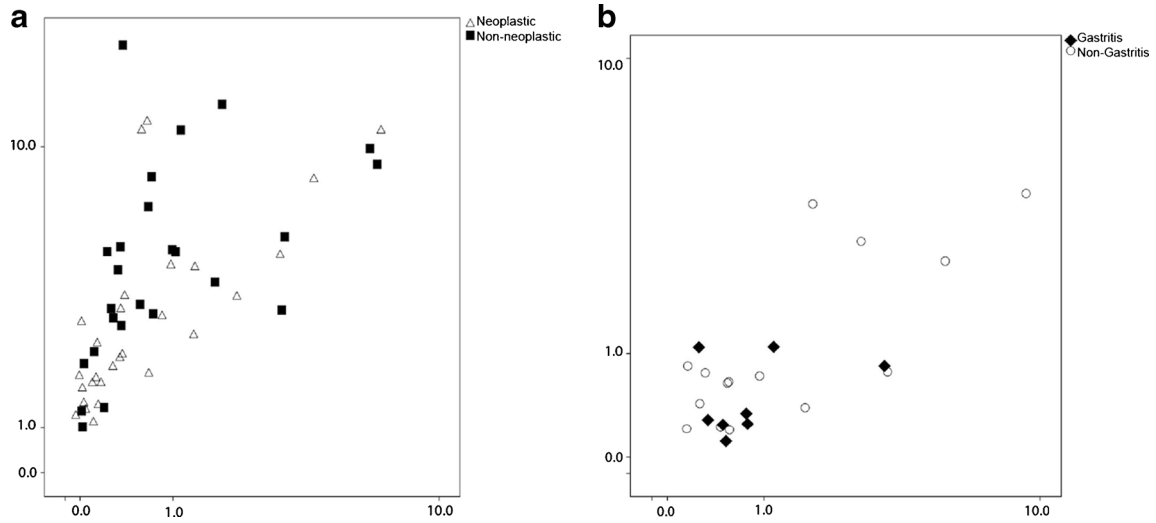

Fig. 2 Correlation between *MBD2* and *MBD3* expression levels. A strong correlation between mRNA expression levels was observed for both neoplastic (*white triangle*) and nonneoplastic groups (*black square*) (**a**); and for the non-gastritis group (*white circle*), but not for the gastritis group (*black diamond*) (**b**)

aberrant methylation of gene promoters in human cancer cell lines [9, 20, 21]; nevertheless, no other study has investigated epigenetic modifications and the mRNA expression of MBD genes in the progression of gastric cancer.

In the current study, we found lower *MBD2* mRNA expression in neoplastic gastric samples compared with the gastric mucosa from noncancer subjects, corroborating previous studies in prostate cancer and other solid tumors [22]. In gastric cancer, reduced mRNA expression levels of *MBD2* have been reported in tumor samples compared with paired nonneoplastic tissue samples [23]. Our findings showed the same results, although the statistical significance was lost after multiple comparison corrections. Taken together, these results suggest that decreased *MBD2* expression is an early event in gastric carcinogenesis and might occur during tumor progression in the gastric mucosa.

MBD2 has been shown to bind to the aberrantly methylated promoter region of p14 and p16 genes and appears to cooperate with the HDACs to promote gene silencing in colon cancer [10]. The promoter hypermethylation of both p14 and

Table 1 *MBD2* and *MBD3* mRNA levels and clinicopathological characteristics of the sample

	n (%)	*MBD2* Median ± IQR	*P* value	*MBD3* Median ± IQR	*P* value
Age (year) (mean ± SD)					
≥50 (67.3±10.0)	19 (67.9 %)	1.0±0.58	0.127	1.14±1.31	0.204
<50 (42.8±4.4)	9 (32.1 %)	0.49±0.55		0.51±0.65	
Gender					
Male	16 (57.1 %)	0.65±0.56	0.169	0.98±1.12	0.887
Female	12 (42.9 %)	1.03±1.19		0.96±0.99	
Histopathology					
Intestinal	20 (71.4 %)	0.65±0.60	0.517	0.81±0.83	0.569
Diffuse	8 (28.6 %)	1.0±0.38		1.24±0.83	
Depth of tumor invasion					
T1	6 (21.4 %)	1.01±0.22	0.466	0.90±0.64	1.000
T2–T4	22 (78.6 %)	0.66±0.71		0.96±1.32	
Lymph node metastasis					
Absent	7 (25.0 %)	0.48±0.65	0.413	0.44±0.80	0.171
Present	21 (75.0 %)	0.95±0.63		1.10±1.26	
Stage					
I–II	16 (57.1 %)	0.90±0.60	0.860	0.87±0.88	0.161
III–IV	12 (42.9 %)	0.82±0.66		1.12±4.33	

Table 2 Number and frequency of methylation on the *MBD2* and *MBD3* promoter regions in gastric cancer tissue (N), adjacent nonneoplastic tissue (ANN), gastritis tissue (G), and adjacent gastritis tissue (NG) groups for each gene

Sample groups	*MBD2*		*MBD3*	
	Total	Methylated (%)	Total	Methylated (%)
N	29	5 (17.80)	29	0 (0.00)
ANN	27	3 (10.71)	28	0 (0.00)
G	25	1 (4.00)	25	0 (0.00)
NG	25	2 (8.00)	21	4 (19.05)

p16 genes have also been described in gastric cancer [24, 25]; however, the mechanisms underlying this epigenetic modification are not clear. Moreover, our group has previously described aberrant hypermethylated promoter regions of genes that might be affected by MBD2 and/or the NuRD complex, such as *IGFBP-3* [26], *FHIT* [27], and *hTERT* [28], in a different subset of patients with gastric adenocarcinoma. MBD2 was proposed to recruit a protein involved in cell growth and differentiation, TACC3, to methylated promoters and to be able to reactivate transcription by favoring the formation of a HAT-containing MBD2 complex, thus switching the repression potential of MBD2 in activation [29]. In addition, MBD2 is known to play a role in the silencing of the *hTERT* promoter region in somatic cells. The upregulation of this gene is a key event for the ability of many cancer cells to overcome the replicative senescence associated with telomere shortening [30]. Taken together, these findings further support the hypothesis that MBD2 might play an important role in the maintenance of the methylation status of carcinogenesis-related genes, and *MBD2* deregulation might lead to alterations in the expression of downstream genes.

Our data show reduced *MBD3* mRNA levels in the gastric cancer samples, although the changes were not significant. To our knowledge, the mRNA expression levels of *MBD3* are described here for the first time in gastric cancer. Decreased *MBD3* mRNA levels have been shown to lead to disassembly of the NuRD complex [31, 32]. In a recent study, Aguilera et al. reported that mice deficient in *MBD3* had an increased susceptibility to colitis-induced tumorigenesis [33]. Nevertheless, the overexpression of *MBD3* mRNA has been described in lung and pancreatic cancer [34–36]. These findings suggest that abnormalities in *MBD3* mRNA levels are a consistent marker of the carcinogenesis process; however, this seems to be cancer type specific.

We did not find any associations between the mRNA levels of *MBD2* and *MBD3* and clinicopathological characteristics. Two independent studies on gastric cancer also showed that *MBD2* mRNA expression levels were not associated with either the clinicopathological features or malignancy potential [23], suggesting that *MBD2* expression does not contribute directly to the clinical features. Moreover, our results also revealed that the NG, G, ANN, and N groups have decreased mRNA levels of *MBD2* according to the alteration level, i.e., the N group presented the lowest and NG the highest *MBD2* expression levels. Thus, the reduced mRNA expression of *MBD2* and *MBD3* in gastric cancer may have a step-specific role in the gastric carcinogenesis process and could be used as biomarkers for the detection or monitoring of cancer development and progression.

Furthermore, a strong correlation between the *MBD2* and *MBD3* mRNA levels was found within the groups, except for the gastritis samples, because both MBD2 and MBD3 are components of the NuRD complex. An increasing number of publications have been addressing the functional differences and similarities between MBD2 and MBD3. MBD2 appears to bind specifically to 5-methylcytosine [10, 37], while MBD3 binds to 5-hydroxymethylcytosine. Moreover, a recent study revealed that whereas MBD2 binds to methylated CpG islands and inactive promoters, MBD3 binds to unmethylated CpG islands and active promoters [38]. Purification of the NuRD complex revealed two different complexes, MBD2–NuRD and MBD3–NuRD [39], and functional analysis showed that MBD2–NuRD, but not MBD3–NuRD, transformed euchromatin into repressed chromatin. Nevertheless, there is evidence that they interact in vivo and in vitro [40], and it was proposed that MBD2 recruits MBD3 to methylated DNA in vitro [31].

The DNA methylation status of both gene promoter regions was also evaluated in this study to verify whether this specific epigenetic modification was responsible for the gene expression alterations observed here. We found an inverse correlation between the *MBD2* expression levels and the promoter methylation frequency in neoplastic tissue, adjacent nonneoplastic tissue, and gastritis samples but not in the non-gastritis samples. Additionally, an interesting finding in this study was the loss of *MBD3* promoter methylation in the G, ANN, and N groups and the presence of methylation only in the NG group, suggesting that the *MBD3* demethylation might occur in a premalignant stage such as gastritis.

Taking a closer look at the analyzed CpGs, we found that the methylated CpGs occurred within the E2F1-binding site in 55 % of all the *MBD2* samples and in 75 % of the *MBD3* samples. The other methylated sites in the *MBD2* promoter did not correspond to any known transcription factor binding sites. In the *MBD3* promoter region, we also observed methylation in the CpGs corresponding to the AhR-Arnt-binding site. Both proteins have roles in the cell cycle, and AhR-Arnt was confirmed to interact in the regulatory region in the E2F1 target gene, followed by the recruitment of downstream activators, including HAT activity in lung cancer cell lines [41]. In gastric cancer, E2F1 overexpression suggests a tumor-suppressing role via the induction of apoptosis [42]. It is not known whether E2F1 or AhR-Arnt have a binding preference

for methylated cytosine, but it has been shown that the E2F1/AhR-Arnt complex can act as an activator for cell proliferation genes and as a suppressor for apoptosis-related genes [41]. This therefore supports our evidence that other epigenetic mechanisms might be involved in the regulation of gene expression [4]. Another point to be considered is that we looked only approximately 500 bp upstream of the transcription start site of each gene. A recent paper has shown that methylated CpGs as well as other modifications of distal upstream regions, gene body, and intergenic regions might also play a crucial role in the regulation of gene expression [43].

miRNA regulation might be involved in *MBD2* downregulation observed in gastric cancer. A study in hilar cholangiocarcinoma showed evidences that miR-373 acts as a negative regulator of MBD2 activity [44], as well as, miR-221* and miR-224-reduced expression contribute to increased levels of MBD2 in colorectal cancer in mice [45]. Indeed, miR-373 upregulation and its oncogenic role by increasing cell proliferation were recently described in gastric cancer [46]. Upregulation miR-221 analyzed in 88 % of gastric tumors [47] might also be a contributing factor for decreased *MBD2* mRNA levels. On the other side, there are no reports of any miRNA regulating MBD3 expression.

In conclusion, the reduced mRNA expression of *MBD2* and *MBD3* could directly or indirectly play a role in the production and maintenance of regional DNA methylation and might be a marker of gastric carcinogenesis. Moreover, the weak inverse correlation between the *MBD2* mRNA expression and its methylation pattern suggests a minor role for *MBD2* methylation in mRNA expression. Therefore, the molecular mechanisms regulating the expression of *MBD2* and *MBD3* should be further investigated in relation to their association with human carcinogenesis. Although DNA methylation has been extensively investigated in cancer, the approach employed in this study allowed us to clarify the role of methylation alterations involved in damage evolution in the gastric tissue and might provide a biomarker for the early diagnosis of this neoplasia.

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Conflict of interests None

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