Promoter Polymorphisms and Methylation of E-Cadherin (*CDH1*) and *KIT* in Gastric Cancer Patients from Northern Brazil

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Abstract. *The aim of this study was to verify genetic and epigenetic alterations in gastric cancer patients from Pará state, northern Brazil. Materials and Methods: Exon 11 of KIT and two promoter polymorphisms (–160 C/A and –347 G/GA) of the E-cadherin gene (CDH1), and their correlation with the promoter methylation status were analyzed. Results: No genetic alterations in KIT were found. Promoter polymorphisms revealed an increased probability of developing gastric cancer, especially of the diffuse-type, in patients carrying –160 A and –347 GA alleles. Analyses of CDH1 methylation suggested a significant difference between hypermethylated and non-hypermethylated samples, with a positive association between the –160 A allele and hypermethylation. Conclusion: Our results suggest that –160 A and –347 GA polymorphisms may increase the chance of developing gastric cancer in the studied population and that –160 A polymorphism seems to be related to the hypermethylation pattern of the promoter region of CDH1.*

Gastric cancer (GC) remains the second most common cancer worldwide, despite a decline in its incidence, due to its poor prognosis and limited treatment options (1, 2). In Brazil, it is the fifth most common kind of cancer, with approximately 21,800 new estimated cases in 2008. Fatalities due to GC were the most frequently reported among cancer patients in Pará state (northern Brazil) during the years of 1999 and 2000 (3). Hence, the city was considered the eleventh in rank of cancer incidence worldwide (4). Yet in 2008, GC was estimated to be the second most common kind of cancer among men and the third among women (5).

Over 95% of all stomach tumors are adenocarcinomas, which are classified according to the site of origin and pathology (6). The widely applied classification of Lauren (7) subdivides stomach neoplasms into intestinal and diffuse types, which have different origins, histology, epidemiology, pathogenesis, genetic profile and clinical outcome (8).

Unlike colon cancer, in which a mutation of the adenomatous polyposis (*APC*) gene is implicated in nearly 90% of cases, GC appears to be initiated by genetic and/or epigenetic alterations (8). Several genes are involved in both types and one of the most studied is that for E-cadherin (*CDH1*).

E-Cadherin is a transmembrane glycoprotein expressed in all epithelial tissues, and which participates in the calcium-dependent interaction between adjacent cells that also appear to have a role in organogenesis and morphogenesis (9, 10). Recently, alterations in its expression, due to polymorphisms and promoter hypermethylation, were associated with tumor progression and invasion in a variety of human cancer types, but mostly in diffuse gastric ones (11). In humans, the *CDH1* gene is located on chromosome 16q22.1, and codifies a mature polypeptide with 728 amino acids (12). This gene is frequently inactivated by genetic alterations, such as loss of heterozygosity (LOH), but also by mutations in its coding region and in the promoter. Polymorphisms at positions –160 C→A and –347 G→GA (numbered from the transcriptional start site) reduce the transcriptional activity of *CDH1*,

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Table I. *Primers used in the present work with their respective annealing temperatures, number of cycles used in PCR reactions, and reference.*

Gene fragment	Primers sequences	Annealing temperature	No. of cycles	Reference
KIT Exon 11	Foward: 5'CCAGAGTGCTCTAATGACTG3'			
	Reverse: 5'AGCCCCTGTTTCATACTGAC3'	50°C	40	24
CDH1 methylation	Foward, Outer: 5'TTTTGATTTTAGGTTTTAGTGAGTTAT3'			
	Reverse, Outer: 5'AATACCTACAACAACAACAACAA3'	52°C	40	26
	Foward, Nested: 5'TGTAGGTTTTATAATTTATTTAGATTT3'			
	Reverse, Nested: 5'ACTCCAAAAACCCATAACTAAC3'	54°C	40	26
CDH1 promoter polymorphisms	Foward: 5'GCCCCGACTTGTCTCTCTAC3'			
	Reverse: 5'GGCCACAGCCAATCAGCA3'	58°C	40	23

although their association with high susceptibility to gastric cancer is controversial (13-16). Epigenetic changes are also associated with the transcriptional silencing of *CDH1* and several studies associated promoter hypermethylation with gastric cancer (11, 17-19).

On the other hand, the proto-oncogene *KIT* encodes a 145-kDa transmembrane glycoprotein that belongs to a type III receptor tyrosine kinase family and regulates cell growth, migration, and survival of several cell types. Mutations of the *KIT* gene have been detected in 20% to 92% of gastrointestinal stromal tumors (GISTs). More than 90% of them are located within the *KIT* juxtamembrane domain (exon 11) (20-22). Due to its high frequency of mutations in GISTs, *KIT* is considered a genetic marker for this kind of disease. However, little is known about *KIT* mutations in GC.

The present work aimed to analyze exon 11 of the *KIT* gene and two promoter polymorphisms, –160 C/A and –347 G/GA, of the *CDH1* gene in gastric cancer patients from Belém, and to assess the correlation of polymorphisms to the methylation status therein.

Materials and Methods

Tumoral and non-tumoral gastric tissue samples were collected from individuals who underwent gastrectomy at Hospital Ofir Loyola and João de Barros Barreto, in Belém (Pará state). Tumor cells were isolated through microdissection and classified according to Làuren (7). Blood samples were obtained at the Laboratório de Análises Clínicas (UFPA) for control purposes in the polymorphism analysis from cancer-free individuals. The control population was age and sex matched with the gastric cancer patients.

All patients signed an informed consent form agreeing with this study, and all procedures were approved by the Ethical Committee of the involved hospitals.

After microdissection, DNA was obtained using QIAamp DNA Mini Kit (Qiagen, Mainz, Rheinland-Pfalz, Germany). A fragment of the promoter region of *CDH1*, covering the –347 and –160 polymorphic positions, and the exon 11 of *KIT* gene were amplified by polymerase chain reaction following the conditions described elsewhere (23, 24). Primer sequences and PCR conditions are described in Table I.

For methylation analyses, a subset of the samples was subjected to DNA modification using sodium bisulfite (25). A fragment with 22 CpGs of the *CDH1* promoter region was amplified using a nested PCR strategy (26) (Table I). Fragments obtained were purified using the phenol-chloroform method (27) and sequenced using an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were aligned with BioEdit v7.0.5 (28). Methylation analyses were run in BiQ Analyzer (29) software. Samples with more than 20% of CpG sites methylated were considered hypermethylated. Information about transcription factor binding sites in the *CDH1* promoter region were obtained using TFSEARCH (30) and TESS (31) websites.

Correlations among polymorphisms, hypermethylation and clinicopathological features were tested with the Chi-square test or G-test, according to the sample group, and with calculation of odds ratios (OR). Hardy-Weinberg equilibrium was also tested. A significance level (α) of 0.05 was adopted for all used tests. All statistical analyses were calculated in BioEstat software, v5.0 (32).

Results

Fifty-eight samples of non-tumoral mucosa from gastric cancer patients and 54 blood control samples (mean age=49.7 years) were used in polymorphism analyses. Clinicopathological characteristics of all patients are described in Table II.

No genetic alterations in exon 11 of the *KIT* gene were found in the sequenced samples, suggesting that, contrary to the situation described for GISTs, this gene is not a good susceptibility marker for gastric carcinoma in our population.

The *CDH1* –160 polymorphism was analyzed in 58 patients and 51 control samples. Frequencies of A and C alleles were 0.2353 and 0.7647, respectively, in the control group and 0.3621 and 0.6379 in the GC patients group. The C/C genotype was the most frequent, being found in 46.55% and 62.75% of patient and control samples, respectively.

Allelic and genotype frequencies for the two groups (patients and controls) are summarized in Table III. Both groups behave according to Hardy-Weinberg equilibrium and no significant difference was observed between them (χ^2=3.297; p=0.1924), although the difference was slightly

Table II. *Clinicopathological characteristics of samples from gastric cancer patients used in the present work.*

Variable	Cases n (%)	Intestinal-type n (%)	Diffuse-type n (%)
Gender			
Male	42 (72.4)	25 (75.8)	17 (68.0)
Female	13 (22.4)	6 (18.2)	7 (28.0)
Unknown	3 (5.8)	2 (6.1)	1 (4.0)
Mean age, years (SD)	56.6 (12.3)	57.6 (9.8)	55.3 (14.9)
Histological type		–	–
Intestinal	33 (56.9)		
Diffuse	25 (43.1)		
Tumor localization			
Antrum	37 (63.8)	23 (69.7)	14 (56.0)
Non-antrum	18 (31.0)	9 (27.3)	9 (36.0)
Unknown	3 (5.2)	1 (3.0)	2 (8.0)
Depth of invasion			
T1	4 (6.9)	1 (3.0)	3 (12.0)
T2/T3/T4	54 (93.1)	32 (97.0)	22 (88.0)
Nodal metastasis			
N0	11 (19.0)	4 (12.1)	7 (28.0)
N1/N2/N3	47 (81.0)	29 (87.9)	18 (72.0)

Table III. *Genotype and allelotype frequencies of E-cadherin (CDH1) promoter polymorphisms in the studied population.*

Polymorphism	Controls n (%)	Cases n (%)	Intestinal-type n (%)	Diffuse-type n (%)
–160 bp				
C/C	32 (62.8)	27 (46.5)	7 (21.2)	9 (36.0)
C/A	14 (27.3)	20 (34.5)	8 (24.2)	12 (48.0)
A/A	5 (9.8)	11 (19.0)	18 (54.6)	4 (16.0)
C allele	0.7647	0.3621	0.3333	0.4
A allele	0.2353	0.6379	0.6667	0.6
	p=0.0903	p=0.0535	p=0.0090*	p=1.0000
NT	3			
–347 bp				
G/G	37 (69.8)	29 (63.0)	17 (68.0)	12 (57.1)
G/GA	11 (20.8)	11 (24.0)	5 (20.0)	6 (28.6)
GA/GA	5 (9.4)	6 (13.0)	3 (12.0)	3 (14.3)
G allele	0.8019	0.7500	0.7800	0.7143
GA allele	0.1981	0.2500	0.2200	0.2857
	p=0.0116*	p=0.0140*	p=0.0370*	p=0.1692
NT	1		4	

NT, Not tested. Asterisks denote samples with Hardy-Weinberg deviation (p<0.05).

greater for A/A genotype (18.97% in patient and 9.8% in control samples).

Comparisons between tumor and control samples and between the two histological types showed no significant association between individuals aged 50 years or less and those older than 50 years.

Considering the presence of the –160 A allele, a difference close to statistical significance was found between tumor and control groups (χ^2=2.866; p=0.0905) and its presence increased the probability of developing GC by two-fold in our population (OR=1.9337, 95% CI=0.8979-4.1643), especially in individuals aged more than 50 years (OR=1.8333; 95%CI=0.7179-4.6816).

A comparison between the histological types of GC revealed that the presence of the A allele increased the chance of developing GC of diffuse type by 23-fold (OR=22.5000; 95%CI=8.7053-58.1542).

Sequences of 46 patient and 53 control samples were obtained for the –347 G→GA polymorphism. Frequencies for G and GA alleles, respectively, were 0.75 and 0.25 in patients, and 0.8019 and 0.1981 in controls. The G/G genotype was the most frequent in both groups, occurring in 69.81% and 63.04% of control and patient samples, respectively. Allelic and genotype frequencies for the two groups (patients and controls) are summarized in Table III.

No statistical difference was observed between patients and controls (χ^2=0.568; p=0.7526) nor between the two histological types of gastric tumors (G=0.6092; p=0.7374). However, patients older than 50 years with the GA allele appear to have more chance of developing GC (1.6-fold; OR=1.6579; 95%CI=0.6023-4.5638) than control individuals. A similar tendency was observed in relation to the diffuse type of tumor (OR=1.5000; 95% CI=0.3783-5.9484).

Table IV. *Clinicopathological characteristics of the studied samples according to their methylation status.*

Variable	Methylated samples	Non-methylated samples	
Nonneoplastic mucosae	9	21	χ^2=0.2860
Neoplastic mucosae	12	21	p=0.5926
Histological type			
Intestinal	5	9	χ^2=0.004
Diffuse	7	12	p=0.9460
C allele	0.5952	0.7381	G=7.5450
A allele	0.4048	0.2619	p=0.02320*
Depth of invasion			
T1	0	1	G=0.9217
T2/T3/T4	12	20	p=0.3370
Nodal metastasis			
N0	1	3	G=0.2670
N1/N2/N3	11	18	p=0.6053

Asterisk indicates p<0.05.

A haplotype analysis revealed no difference between cases and controls. However, individuals with the A/GA haplotype had an increased risk of developing GC by 2-fold (OR=2.0952; 95% CI=0.6938-6.3273).

None of the polymorphisms or haplotypes tested had any significant correlation with nodal invasion, site of tumor or tumor depth, although patients with the –347 GA allele had

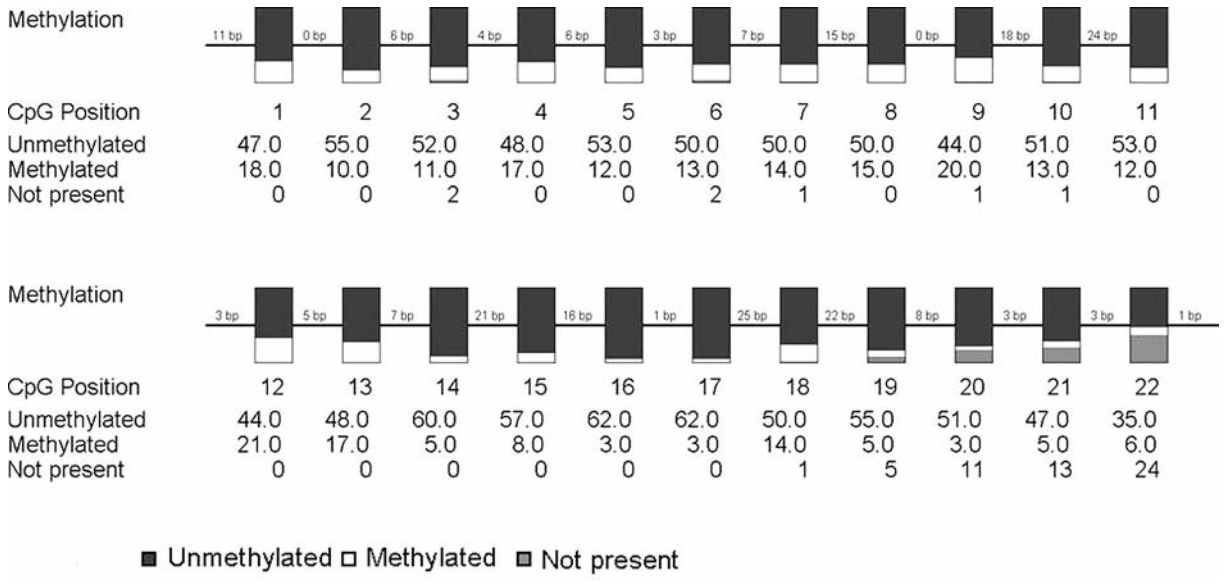

Figure 1. *Schematic diagram showing the methylation pattern of each CpG in the E-cadherin (CDH1) promoter region analyzed in the present study.*

a higher chance of developing tumors to an advanced stage (T2-T4) (OR=1.8462; 95% CI=0.1765-19.3065).

Tumoral samples (N=33) had their *CDH1* methylation pattern analyzed and compared with samples of non-tumoral gastric tissues (N=30). Hypermethylation was observed in 12 (36.36%) tumor samples and nine (29.03%) non-tumoral gastric tissues. The great majority of hypermethylated samples (75%) were from patients over 50 years old and from the diffuse type of GC (58.33%). The frequency of the A allele was 0.4048 and 0.2619 in methylated and non-methylated samples, respectively.

The relationship between hypermethylation and promoter polymorphism –160 C/A was investigated and is summarized in Table IV. A significant difference was observed between hypermethylated and non-hypermethylated samples (G=7.5450; p=0.02320), with a positive association between the A allele and hypermethylation (OR=3.600; 95% CI=1.1921-10.8720).

In order to identify the CpGs sites that are more susceptible to hypermethylation in the promoter region of *CDH1* gene, a quantitative analysis of the 22 CpGs sites was carried out using bisulfite sequencing methodology. We observed a higher methylation rate at the 5' end of the sequenced fragment, covering 13 CpGs (Figure 1), suggesting that some factor might favor hypermethylation in this region. Hence, we looked for transcription factor binding sites in the *CDH1* promoter, using the non-converted region (Genbank accession number DQ090940) that includes the –160 polymorphic position studied by us. There are three Sp1-binding sites in the studied region, two of them near to our target site. Potential binding sites for RAR-β, ER-α, AP-1, StuAp and CF-1 were found despite the polymorphism in the –160 position. However, the –160 C→A transversion eliminates the binding site for CF-1 and creates putative sites for another two transcription factors: RC2 and MCBF (Figure 2). Whether these binding sites have some influence in gene silencing, favoring a hypermethylation pattern is not clear and expression assays are needed to clarify this issue.

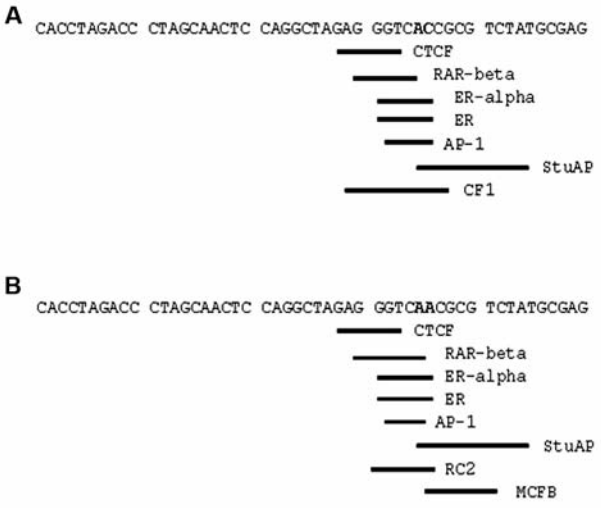

Figure 2. *Putative transcription factor binding sites in the promoter region of E-cadherin near the –160 polymorphic site, according to the promoter sequence. A: –160 C allele, and B: –160 A allele. In bold, the –160 position.*

Discussion

The absence of *KIT* mutations in GC patients and controls was also found in samples of Finnish patients with gastric adenocarcinomas (33). Although rare, synchronous occurrence of GISTs and GC were described in literature (34-37). A high incidence of microscopic GISTs (35%) was also described (38), suggesting that small GISTs developed much more frequently in the stomach than their estimated clinical incidence suggests (38). Neverthless, some studies in large groups of colorectal cancer patients identified a small group with altered KIT expression, suggesting that anti-KIT therapy could be used together with chemotherapy in those patients (39, 40). Thus, the investigation of other gene regions and expression in a larger sample could clarify this subject in GC patients in our region.

CDH1 promoter polymorphisms are frequently analyzed in GC tumors, although its association with tumorigenesis is still controversial. The frequency of the –160 A allele in healthy individuals varies in different geographic areas, ranging from 61% and 43.3%, in China and Italy, respectively, to 23.3% and 14.3% in the United Kingdom and Korea, respectively (41). Meanwhile, the frequency of A/A homozygotes ranges from 44% and 18.9% in China and Italy, respectively, to 3.4% and 0% in the United Kingdom and Japan, respectively.

In the present study, the control group with an allelic frequency of 23.53% for –160 A is close to that observed in the United Kingdom, and the A/A homozygote frequency (9.8%) is similar to that found in a large European sample (9.5%), in Taiwanese (9.7%), in Germans (11.9%) and Portuguese (8.2%) (42-44).

Studies of *CDH1* –160 C→A promoter polymorphism in GC populations described a prevalence of the C/C genotype, with frequencies ranging from 39.2% to 75% (13, 16, 43-45). The C/C genotype frequency obtained by us (46.55%) is similar to those described for Europeans (48.6%), Taiwanese (47.3%), Canadian (46.2%) and Portuguese (46.2%) GC patients (42-44).

However, our results showed the A/A genotype frequency (18.97%) to be higher than those for the great majority of the studied populations and closer to those described for Taiwanese (21%) and Italian patients with diffuse type GC (18.9%) (15, 46).

No association between the A allele and GC (including age, tumor site, stage, nodal invasion and histology) was found (13, 16, 42, 44, 46-48). On the other hand, a significant reduced risk of GC in individuals with –160 A allele was also suggested (43).

Contrary to that view, a study including only diffuse type GC showed a positive association between the A allele and an increased risk of diffuse-type GC development (15). Recently, a meta-analysis suggested that the –160 A allele is an ethnicity-dependent risk factor for GC, and that it may be a marker for genetic susceptibility rather than a prognostic marker (41). Another study obtained a heterogeneous, ethnicity-dependent result where the –160 A allele seems to decrease GC risk in Asians, and to increase it in Caucasians (49).

The –347 G→GA was first described by Shin *et al*. (50, 51) who observed a decrease of the transcriptional activity of *CDH1* with the GA allele, and the association of this allelotype and colorectal cancer. Only one study on this polymorphism and GC has been conducted so far. Association between the GA allele and an increased risk (1.45-fold) of GC was reported in a Chinese population, similar to the results obtained in the present study (16). The authors found a significant increase risk of GC in individuals with –160 C/–347 G and –160 C/–347 GA haplotypes, which differs from the results of our work, where a higher risk of GC, especially in advanced stages, was seen in those with the –347 GA allele.

Differences in ethnic composition between our population and those from the literature could explain the allelic and genotype frequencies obtained. The borderline association found by us between the A allele and gastric cancer, especially of the diffuse type, may suggest that it acts as a susceptibility marker for GC in our population. However, a larger sample size must be analyzed to confirm these results.

It is widely known that epigenetic alterations are characterized as the inheritance of information based on gene expression levels, achieved by changes in the chromatin configuration assumed by a DNA region. Such configuration can be due to methylation, phosphorylation, acetylation and sumoylation processes (18).

Methylation of a particular gene in certain tumor types can occur because its inactivation confers a selective clonal advantage on the tumor cell (18). *CDH1*, similar to a growing list of important genes in cancer, is clearly targeted by both epigenetic and genetic mechanisms of inactivation during tumor development (19).

Frequencies of *CDH1* methylation show great variation in populations. In Hong Kong, methylated frequencies range from 58% in primary gastric tumors, to 65% in metastatic ones, with a positive association between methylation and depth of tumor invasion and nodal metastasis (52). On the other hand, higher hypermethylation incidences (80.8% and 75.9%, respectively) with no association with any clinicopathological feature were reported (53, 54). In the Japanese, frequencies ranged from 21.9% (55) to 95.45%, in EBV-positive patients (56), and no association between methylation and tumor depth and nodal invasion were found (57, 58). These differences may be explained by the different tumor stages analyzed (57). Association between diffuse histotype and promoter methylation was reported only by one study (58), while the correlation of methylation with the age of the patients was described by others (59, 60).

Recently, a frequency of 31.7% of methylation in GC samples, with a strong correlation with *Helicobacter pylori* infection was described (61).

Frequencies also had a great variation (45% to 67%) in different studies with Chinese patients, with a higher frequency in tumors of the diffuse type and with older age of patients (62-64). In contrast to observations in Asia, frequencies from European studies are lowest (51% in Portuguese, 54% in Italians and 30% in Germans), with no correlation with any clinicopathological features, except in Italians, where a positive association was observed between hypermethylation and diffuse type GC (65-67).

The hypermethylation values found in our study are lower than those described for Europeans and close to some reported for Asian populations, and very discrepant from those reported elsewhere for the same population (98.7% and 92.3%, for tumoral and non-tumoral samples, respectively) (68). This variation may be explained by differences in the applied methodology, as the latter used methylation-specific PCR (MSP) to access data about DNA methylation in the *CDH1* promoter. MSP is a powerful and sensitive technique used to detect hypermethylation based on primer annealing during the PCR. However, such a qualitative technique is only able to detect methylation present in more than one CpG in the primer set and is susceptible to false-positive results (69, 70).

In our study, *CDH1* hypermethylation was analyzed in a quantitative manner, using bisulfite sequencing, so we could individually detect sites more susceptible to methylation in the fragments (69, 71, 72). Quantitative studies are important to detect core regions susceptible to hypermethylation. The methylation status of a CpG island is sometimes assumed to be homogeneous (either entirely methylated or unmethylated), but it varies among the regions within a CpG island, and transcription is only consistently repressed when the region covering the transcription start site (core region) is methylated. Hence, in order to identify genes that are silenced by methylation, studies should be focused on methylation of the core region within a promoter CpG island, in order to avoid obtaining false positives (73). Moreover, we only considered samples with 20% or more CpG sites methylated as being hypermethylated, which according to the literature is correlated to complete gene silencing (17). Such a strict criterion is likely responsible for the difference observed in methylation rates in the same population.

Due to its strong association with the aging process and the high similar frequency of methylation in four-step lesions leading to gastric carcinogenesis (including normal mucosa), *CDH1* was considered as a Type A (aging) gene (74, 75). However, a recent study (76) classified *CDH1* as Type M (mixed) gene, because of its compatibility with age- and cancer-associated features. In the present study, we observed a positive association between hypermethylation and older age, as a significant correlation between hypermethylation and the A allele (which increases the risk of develop GC). These results are in agreement with the proposal of the recent classification of *CDH1* as a Type M gene in gastric carcinogenesis.

With regard to the high proportion (29.03%) of hypermethylation in non-tumoral samples observed in our population, exogenous and endogenous factors may explain this result. Ethnicity and environmental factors, such as *Helicobacter pylori* and Epstein-barr (EBV) infections, chronic gastritis, intestinal metaplasia and dietary factors (74, 77-80) are known to influence hypermethylation in non-neoplastic gastric tissues.

Here we isolated a fragment of the *CDH1* minimal promoter region, responsible for the expression of the gene (81). The higher CpG site methylation in the 5' portion found by us is in agreement with other works (26), which cited this region as being responsible for the down-regulation of *CDH1*. A hypomethylated region observed by us, compassing CpGs 14 to 17, is adjacent to an Sp1-binding site (17), an element that confer a protection against methylation, which could explain this observation, although the mechanism is still not elucidated (82-84).

A correlation between the –160 A polymorphism and hypermethylation was also observed here. To assess the relationship between the polymorphism and the methylation status, we looked for changes in the transcription factor-binding sites that could favor methylation. None of the predicted binding sites created or eliminated by the presence of –160 A seem to explain the observed correlation. However, the presence of a mutation in the promoter sequence and the hypermethylation of the same region fits Knudson's two-hit hypothesis, with methylation usually considered as the second hit (17, 85).

In conclusion, our results suggest that –160 A polymorphism may increase the chance of developing GC in the studied population, and patients with –347 GA polymorphism have a higher chance of developing tumors of an advanced stage. Thus, –160 A polymorphism seems to be related to the hypermethylation pattern of the promoter region of *CDH1* gene. We also suggest that the 5' portion of the *CDH1* promoter region analyzed in the present work is more prone to methylation than the rest of the fragment, suggesting its important role in the silencing of the gene. Nevertheless, a wider sample size is necessary to better assess such findings.

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