Virchows Arch (2010) 457:627–633
DOI 10.1007/s00428-010-0996-3

ORIGINAL ARTICLE

MTHFR C677T polymorphism and differential methylation status in gastric cancer: an association with *Helicobacter pylori* infection

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Received: 25 June 2010 / Revised: 1 October 2010 / Accepted: 5 October 2010 / Published online: 19 October 2010
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Abstract *MTHFR* C677T and *Helicobacter pylori* infection are believed to play critical roles in the DNA methylation process, an epigenetic feature frequently found in gastric cancer. The aim of this study was to verify the associations between the *MTHFR* C677T polymorphism and the methylation status of three gastric cancer-related genes. The influence of *H. pylori* strains was also assessed. DNA extracted from 71 gastric tumor samples was available for *MTHFR* C677T genotyping by PCR-RFLP, promoter methylation identification by MS-PCR and *H. pylori* detection and posterior subtyping (*cag*A and *vac*A genes) by PCR. In the distal tumors, a positive correlation was found between the methylation of *CDKN2A* and the allele T carriers (r=0.357; p=0.009). Considering the eldest patients (age≥60 years old), this correlation was even higher (r=0,417; p=0.014). *H. pylori* infection by highly pathogenic strains (*cag*A+/*vac*As1m1) was also found correlated to promoter methylation of *CDKN2A* and the allele T carriers in distal tumors (r=0.484; p=0.026). No significant correlation was verified between *MTHFR* C677T genotype and promoter methylation status when we analyzed the general sample. DNA methylation in *CDKN2A* associated to the *MTHFR* 677T carrier is suggested to be a distal tumor characteristic, especially in those 60 years old or older, and it seems to depend on the infection by *H. pylori cag*A/*vac*As1m1 strains.

Keywords *MTHFR* C677T · DNA methylation · *Helicobacter pylori* · Gastric cancer

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Introduction

Gastric adenocarcinoma is one of the most frequent malignancies worldwide and it is globally the second leading cause of cancer-related deaths [1]. Brazil is one of the countries with a higher incidence, mainly in the Southeast and Northeast regions [2]. Gastric cancer has multifactorial etiology, being caused by environmental, genetic and epigenetic factors, which may interact to the tumor promotion and progression [3]. Epidemiological studies have shown an association between disturbances of folate metabolism and increased risk of gastric cancer, including low intake of folate, low levels of folate in blood or genetic factors affecting folate metabolism, such as polymorphisms in folate-metabolizing genes [4–6].

Methylenetetrahydrofolate reductase (MTHFR) acts centrally in folate metabolism, catalyzing the 5-methylenetetrahydrofolate synthesis reaction, the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine. This pathway leads to the production of *S*-adenosylmethionine, the universal donor of methyl radical in human, which is required for DNA methylation [7]. Thus, polymorphisms that reduce the MTHFR activity could influence the status of DNA

methylation and would explain individual susceptibility to gastric cancer [8]. A C/T nucleotide change at 677 position of the *MTHFR* gene (*MTHFR* C677T), resulting in an alanine-to-valine substitution in the MTHFR protein, was found to produce a thermolabile variant of the MTHFR and significantly reduces the enzyme activity [9]. Furthermore, a recent meta-analysis carried out by Boccia et al. reported a significant positive correlation between gastric cancer and the *MTHFR* 677TT genotype [10].

DNA methylation is an important epigenetic feature of DNA that plays critical roles in gene regulation and cellular differentiation mechanisms. Methylation of promoter, especially in the CpG islands, culminates in gene silencing. Gastric cancer is one of the human tumors with higher number of silenced genes by methylation of their CpG islands [11]. Currently, the available data about the role of *MTHFR* C677T polymorphism in relation to the aberrant DNA methylation in gastric cancer is inconsistent and incomplete. It has been suggested that this polymorphism plays a dual role on the methylation status of promoter regions depending on the target gene [8, 12].

Another important etiological factor in gastric cancer is *Helicobacter pylori* infection. Bacterial virulence factors are characteristics present in some bacteria which enable them to cause disease. These are, mainly, vacuolating cytotoxin production associated to certain types of *vacA* (vacuolating cytotoxin gene A) alleles and *cagA* (cytotoxin associated gene product A) [13]. *H. pylori* strains expressing *cagA* and more active forms of *vacA*, such as the s1m1 genotype, are more likely to cause disease [14].

Despite of some advances, the molecular mechanisms associated with *H. pylori* in gastric carcinogenesis remains poorly understood. One of the promoting effects has been attributed to the induction of inflammation and aberrant methylation. It has been reported that frequencies or levels of CpG islands methylation of certain genes correlate with *H. pylori* infection, histological or serological severity of gastritis, and gastric cancer occurrence [12]. The observation that *H. pylori*-induced methylation is reversed after *H. pylori* eradication therapy supports this hypothesis [14]. Thus, the aim of this study was to verify the associations between the *MTHFR* C677T polymorphism genotype and the DNA methylation of three candidates genes (*COX-2*, *CDKN2A*, and *HMLH1*), largely associated with gastric carcinogenesis, and the influence of *H. pylori* strains in this process.

Materials and methods

Clinical specimens

The present study was approved by the Hospital Ethics Committee in the Federal University of Ceará and all subjects signed the informed consent form before inclusion. Samples from 71 patients with gastric adenocarcinoma who had undergone gastrectomy were collected from Walter Cantídio University Hospital and Santa Casa de Misericórdia Hospital, both located in Fortaleza, the Ceará state capital. The histological classification was done according to Lauren's classification by our team of pathologists.

DNA extraction

Genomic DNA was extracted from frozen tumor tissue using the cetyltrimethyl ammonium bromide (CTAB) technique, adapted from Foster and Twell [15]. The DNA extraction was done only in fragments that showed more than 80% of tumor cells. The DNA quality was analyzed by 1% agarose gel electrophoresis and the amount was determined using the NanoDropTM® 3300 fluorospectrometer (Wilmington, DE, USA).

Sodium bisulfite treatment and methylation-specific PCR (MS-PCR)

Extracted DNA of tumor tissue was modified by sodium bisulfite to determine the methylation status of the *CDKN2A, HMLH1*, and *COX-2* genes by MS-PCR, as previously described by Ferrasi et al. [16]. The primers targeted to the studied promoter gene regions are described in Table 1. PCR was performed in 25 μL reaction volume, containing 1× Platinum *Taq* buffer, 3.0 mM $MgCl_2$ (*CDKN2A*) or 1.5 mM $MgCl_2$ (*HMLH1* and *COX-2*), 0.4 mM of each dNTPs, 0.64 μM (*CDKN2A*), 0.24 mM (*HMLH1*) or 0.4 mM (*COX-2*) of each primer set, 1U of Platinum *Taq* DNA Polymerase® (Invitrogen, Foster, CA, USA), and 1 μL of treated DNA. DNA methylated in vitro by Sss-I methylase® (New England Biolabs, Beverly, MA, USA) was used as a positive control. Water and DNA from peripheral lymphocytes of healthy donors were used as negative controls. The PCR products were separated in 6% non-denaturing polyacrylamide gel and subsequently submitted to silver staining (Fig. 1).

For confirmation of the reaction specificity, MS-PCR products from *CDKN2A*, *HMLH1*, and *COX-2* genes analyzed were cloned with a TOPO TA Cloning Kit® (Invitrogen, California, USA) and both the methylated and unmethylated PCR products were sequenced using an ABI PRISM® BigDye Terminator v.3.0 Cycle Sequencing Kit® (Applied Biosystems, Foster, CA, USA) and ABI Prism 3100 DNA Sequencer® (Applied Biosystems).

Determination of MTHFR C677T genotypes

MTHFR C677T genetic polymorphism was detected by PCR-RFLP as previously described by Frosst et al. [17].

Table 1 PCR primer sets used for MS-PCR

Gene	Primer sequences (5′ - 3′)		Annealing (°C)	Size of PCR product (bp)	Ref.
	Forward	Reverse			
CDKN2A	M TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	70	150	[32]
	U TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	70	151	
HMLH1	M TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	66	153	[33]
	U TTTTGATGTAGATGTTTTATTAG GGTTGT	ACCACCTCATCATAACTACCCACA	64	124	
COX-2	M TTAGATACGGCGGCGGCGGC	TCTTTACCCGAACGCTTCCG	59	161	[34]
	U ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA	65	171	

M methylated; *U* unmethylated

The PCR products of 198 bp were digested with restriction endonuclease *Hinf* I, producing a 175 and a 23 bp fragments if the polymorphism was present. The digestive products were resolved by electrophoresis in 6% polyacrylamide gel with silver staining. Random samples were reanalyzed for laboratory procedures control (Fig. 2).

Detection of *H. pylori* and vacA alleles and the presence of cagA gene

H. pylori infection was detected by PCR of the *ure*C gene. The primers for the *H. pylori* studied genes and their annealing temperature are described in Table 2. For confirmation of the reaction specificity, PCR products from *ure*C gene were cloned with TOPO TA Cloning Kit® (Invitrogen, California, USA) and sequenced using the ABI PRISM® BigDye Terminator v.3.0 Cycle Sequencing Kit® (Applied Biosystems, Foster, CA, USA) and ABI Prism 3100 DNA Sequencer® (Applied Biosystems).

The *ure*C, *cag*A, *vac*A s1/s2, *vac*A m1/m2 genes were amplified in a 25-μL volume containing 2.5 μL of 10× PCR buffer® (Invitrogen, Cergy Pontoise, France); 1% Tween 20, 1.5 mM of $MgCl_2$ (Invitrogen), 200 μM (each) of dNTPs (Invitrogen), 1U of Platinum *Taq* polymerase® (Invitrogen); 0.4 μM (*ure*C, *cag*A, *vac*A s1/s2, *vac*A m1), 0.3 μM (*vac*A m2) for each primer and 1 μL of DNA. DNAse-free water was used as negative control. The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining (Fig. 3). v*ac*A and *cag*A genes were considered positive when a specific fragment was detected (Table 2). Random samples were reanalyzed to confirm the results. DNA preservation has been confirmed by amplification of different genes in other approaches under study in our laboratory.

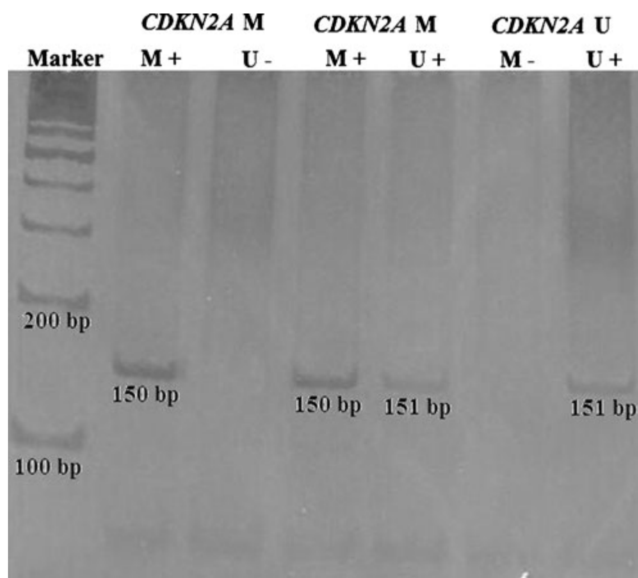

Fig. 1 Representative electrophoresis of the *CDKN2A* MS-PCR in 6% polyacrylamide gel with silver staining

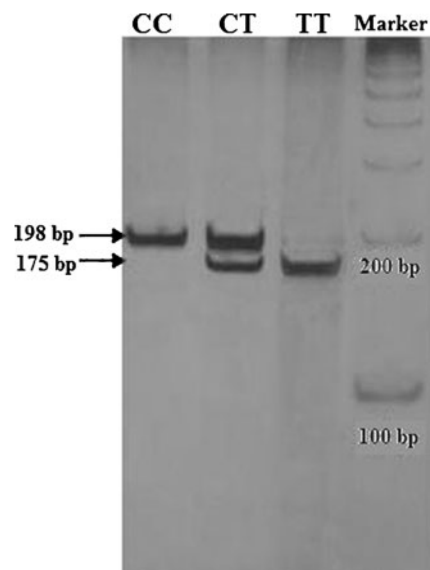

Fig. 2 Representative electrophoresis of the *MTHFR* C677T polymorphism in 6% polyacrylamide gel with silver staining

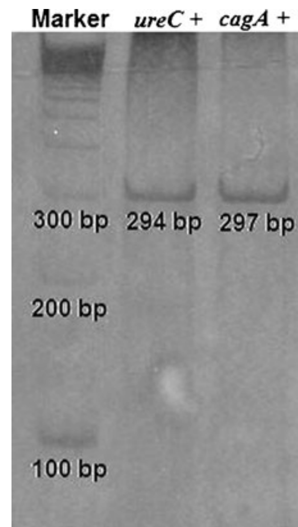

Fig. 3 Representative electrophoresis of the *ure*C and *cag*A genes amplification in 6% polyacrylamide gel with silver staining

Statistical analyses

These were carried out using the statistical program SPSS® version 15.0 (Chicago, IL, USA). Statistically significant differences were evaluated by the chi-square test (χ^2). Correlations were analyzed by Spearman's rank correlation coefficient. The results were considered statistically significant when p values were less than 0.05.

Results

Study population

Among the 71 cases analyzed, 45 (63.4%) were males and 26 (36.6%) females. The average age was 64.2, ranging from 23 to 92 years old. The most frequent site was the gastric non cardia [52/71 (73.2%)×19/71 (26.8%)]. Intestinal and diffuse subtypes presented 43 (60.5%) and 28 (39.5%) frequencies, respectively.

Frequency of promoter methylation of three CpG islands and MTHFR genotypes

All 71 tumor samples were available for MS-PCR analysis and for *MTHFR* genotyping. CpG island methylation was found in 30 of the tumors samples (42.2%) for *CDKN2A*, 38 (53.5%) for *COX-2* and 22 (30.9%) for *HMLH1*. The *MTHFR* C677T genotype shows similar frequency for the homozygous CC (34/71; 47.8%) and heterozygous CT (31/71; 43.6%) carriers. Homozygous carries for *MTHFR* 677TT was found in only 8.6% of the patients.

Association between MTHFR C677T, promoter methylation and clinicopathological parameters

The association of each *MTHFR* C677T genotype to the promoter CpG islands methylation status is shown in Table 3. No significant correlation was found between the promoter methylation for all the studied genes, age (cutoff of 60 years old), histological subtypes (intestinal and diffuse), and *MTHFR* genotype (Table 3).

When the tumors were analyzed according to its localization, in the distal tumors a positive correlation was found between the promoter methylation of *CDKN2A* and the allele T carriers (CT and TT genotypes; r=0.357, p=0.009) (Table 4). Considering the patients age with 60 years old or older, this correlation was even higher (r=0.417, p=0.014).

Influence of *H. pylori* genotype in the methylation process

H. pylori infection was present in 68 of the patients (95.8%). Among them, 31 (45.5%) were *cagA*+/*vacA*s1m1. As most of the patients were *H. pylori* positive, we verify if there was an association between the *H. pylori* strains and the *MTHFR* genotype in gene methylation, considering clinicopathological parameters. From these analysis, we

Table 2 PCR primer sets used for genotyping *H. pylori*

Gene	Primer sequence	Annealing (°C)	Size (bp) of PCR product	Reference number
ureC	F—5′ AAGCTTTTAGGGGTGTTAGGGGTTT 3′ R—5′ AAGCTTACTTTCTAACACTAACGC 3′	55	294	[35]
vacA				
s1/s1	F—5′ ATGGAAATACAACAAACACAC 3′ R—5′ CTGCTTGAATGCGCCAAAC 3′	55	259/286	[36]
m1	F—5′ GGTCAAAATGCGGTCATGG 3′ R—5′ CCATTGGTACCTGTAGAAAC 3′	55	290	
m2	F—5′ GGAGCCCCAGGAAACATTG 3′ R—5′ CATAACTAGCGCCTTGCAC 3′	52	192	
cagA	F—5′ ATAATGCTAAATTAGACAACTTGAGCGA 3′ R—5′ TTAGAATAATCAACAAACATAACGCCAT 3′	56	297	[37]

F forward; *R* reverse

Table 3 Association between the MTHFR C677T genotype, the clinicopathological parameters, and the promoter methylation status for the three studied genes

Variables	MTHFR genotype			Alelle T carriers
	CC	CT	TT	
Sex				
Male	22	18	5	23
Female	12	13	1	14
Age				
<60	13	7	2	9
≥60	21	24	4	28
Lauren's histotype				
Diffuse	13	13	2	15
Intestinal	21	18	4	22
Anatomical site				
Proximal	9	8	2	10
Distal	25	23	4	27
CDKN2A				
Methylated	23	15	3	18
Unmethylated	11	16	3	19
COX-2				
Methylated	17	13	3	16
Unmethylated	17	18	3	21
HMLH1				
Methylated	24	21	4	25
Unmethylated	10	10	2	12

found a high association between the promoter methylation of *CDKN2A* and the *MTHFR* 677T carrier (r=0.484, p=0.026) in the distal tumors infected by *H. pylori cagA*+/*vacA*s1m1.

Association between number of methylated CpG islands and MTHFR genotype

To investigate whether the *MTHFR* genotype might be associated with methylation of multiple CpG sites we grouped all the 71 subjects according to the numbers of methylated genes: none, 1, 2, and 3. No promoter methylated gene was present in 20 (28.2%) of the cases while the presence of 1, 2, and 3 was observed in 19 (26.8%), 25 (35.2%), and 7 (9.8%) of the cases, respectively. The mean number of methylated CpG sites according to the *MTHFR* genotype was 1.12 for CC, 1.42 for CT, 1.33 for TT and 1.40 for T carrier. No significant association was found between *MTHFR* genotype and number of methylated CpG sites and no statistical difference was verified between the number mean of methylated CpG sites over the different *MTHFR* genotypes.

Discussion

The relationship between the hypermethylation of tumor suppressor genes or DNA repair genes with the gastric carcinogenesis process has been pointed in many studies; however, the factors involved in the induction of this process are still unknown [18, 19]. Some studies indicate that DNA methylation depends on environmental factors such as *H. pylori* infection and may also vary according to the presence of polymorphisms involved in this process as those of enzymes involved in the folate cycle [8, 12, 13]. However, there are only few studies focusing these aspects and they present controversial results. So that, in the present study, the association between the methylation status of *CDKN2A*, *HMLH1*, and *COX-2,* the *MTHFR* C677T polymorphism and the influence of high pathogenic *H. pylori* strains in gastric cancer were analyzed.

The first analysis focused the association between the presence of promoter methylation and the *MTHFR* C677T polymorphism. Some studies demonstrated that genetic and epigenetic changes in gastric carcinoma differ depending on the tumor localization and on its histological subtype [20, 21]. Based on that, the data were analyzed according to these aspects. Although no difference was observed regarding to histological subtype, differences were identified according to the tumor anatomic site. In the distal tumors, an unexpected positive correlation was found between the CpG island methylation of *CDKN2A* and the allele T carriers. In fact, this correlation was even higher when we considered the 60 years old or older patients. Tahara et al. [12] also observed a similar correlation between the hypermethylation of *CDKN2A* and the *MTHFR* 677TT genotype, but these authors did not associate it to the tumor anatomic site. Both ours and Tahara et al.'s studies disagree with Weng et al. [22],

Table 4 Association between MTHFR polymorphism and methylation status of three promoter CpG islands on the distal tumors

Variables	MTHFR genotype			Alelle T carriers
	CC	CT	TT	
CDKN2A				
Unmethylated	6	14	2	16
Methylated	9	9	2	11*
COX-2				
Unmethylated	13	14	2	16
Methylated	12	9	2	11
HMLH1				
Unmethylated	10	9	1	10
Methylated	15	14	3	17

*p=0.009

which found a promoter hypermethylation of *CDKN2A* in patients with the *MTHFR* 677CC genotype. Various studies have been associating the *MTHFR* C677T polymorphism to gastric cancer risk in a specific anatomic localization. Wang et al. [23], evaluating the association of *MTHFR* C677T and thymidylate synthase promoter polymorphisms with genetic susceptibility found a gastric cardia adenocarcinoma risk related to the *MTHFR* 677TT and 2R2R genotypes. Likewise, Miao et al. [5] observed that subjects with the *MTHFR* 677TT variant genotype had a twofold increased risk of gastric cardia adenocarcinoma. These studies suggested that the common functionally polymorphism *MTHFR* C677T plays a substantial influence in the gastric cardia adenocarcinoma carcinogenesis. However, both studies did not evaluate the methylation pattern of specific genes associated to this polymorphism. Related to methylation status associated with tumor region there are some controversy. Vo et al. [24] found no distinct methylation status for *CDKN2A* regarded to the tumor localization, while recent studies carried out in Turkey [25] and Germany [26] correlate methylation of promoter *CDKN2A* to cardiac tumors, but the *MTHFR* C677T polymorphism influence was not assessed. We suggest that in distal tumors, the silencing of tumor suppressor genes by their promoter CpG islands methylation, especially when influenced by *MTHFR* polymorphism, might be an important carcinogenic pathway.

It has been well established over the literature that, in general, the methylation status of promoter CpG islands tends to increase with age [27]. From that standpoint, we found that the correlation between methylation of *CDKN2A* and *MTHFR* 677T carrier subjects in distal tumors is higher in an older generation (60 years old or older). It seems that the *MTHFR* polymorphism may have a greater influence on the methylation status as time goes by. Additionally, in the present study, *MTHFR* C677T polymorphism does not act homogeneously over all the studied genes. *MTHFR* 677TT genotype seems to be related to the hypermethylation of *CDKN2A*; on the other hand, it does not show an involvement regarding to *HMLH1* and *COX-2* methylation status. Some studies have been reporting this dual behavior of *MTHFR* C677T polymorphism regarding to methylation or unmethylation of promoter regions depending on the target gene [8, 12]. Our data suggests that this differential methylation status influenced by *MTHFR* C677T genotype depends on the tissue, specifically on the tumor localization. Probably the induction of the methylation process of some genes, like *HMLH1* and *COX-2,* is independent on the *MTHFR* C677T polymorphism.

Although the hypermethylation of promoter regions in genes involved on gastric carcinogenesis has been well accepted and some studies associate it with *MTHFR* C677T polymorphism, the influence of *H. pylori* in this process is still not understood. In the present study, the infection by a highly pathogenic *H. pylori* strain (*cagA*+and *vacA* s1m1 genotype) was associated to the methylation of *CDKN2A* in the distal stomach in patients with *MTHFR* 677TT genotype.

Recently, there have been reports showing that *H. pylori* infection is the most predisposing factor for gene methylation in the stomach [28–30]. It is well known that *H. pylori* induces chronic inflammation, resulting in down-regulation of the some genes that maintain gastric mucosal homeostasis [14]. Also, the inflammation induced by *H. pylori* infection seems to promoter methylation of certain genes [31], which could justify an increased risk of tumor formation, mainly associated with the risk of noncardia gastric cancer.

In conclusion, the DNA methylation in promoter regions of *CDKN2A* associated to the *MTHFR* 677T carrier is suggested to be a distal tumor characteristic, especially in those 60 years old or older, and depends on the infection by *H. pylori cag*A/*vac*As1m1 strains. More studies with large sample size involving the folate enzymes polymorphisms, DNA methylation and *H. pylori* infection are needed to extend our understanding of gastric carcinogenesis.

Conflict of interest statement We attest the inexistence of conflicts of interest, corporate involvement, or patent holdings.

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