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Short Communication

Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms and gastric cancer susceptibility in a population of Northern Brazil

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ABSTRACT. The folate metabolic pathway, which is involved in DNA synthesis and methylation, is associated with individual susceptibility to several diseases, including gastric tumors. In this study, we investigated four polymorphisms [thymidylate synthase enhancer region, single nucleotide polymorphism thymidylate synthase 5' (TS5'), TS3' untranslated region, and methylenetetrahydrofolate reductase (MTHFR) 677C> T] in 2 genes related to the folate pathway, TS and MTHFR, and their possible association with the risk gastric cancer development in a population from

Pará state, Brazil. For the TS enhancer region, TS3' untranslated region, and single nucleotide polymorphism TS5' polymorphisms, no significant results were obtained. For the MTHFR 677C>T polymorphism, TT genotype carriers had a higher risk of developing tumors in the antrum (P = 0.19 *vs* CC and P = 0.02 *vs* CT) and intestine (odds ratio = 4.18, 95% confidence interval = 0.66-26.41; P = 0.252 *vs* CC and odds ratio = 2.25, 95% confidence interval = 0.32-15.75; P = 0.725 *vs* CT). Those carrying at least 1 T allele had an increased risk of lymph node metastasis (odds ratio = 3.00, 95% confidence interval = 0.88-10.12; P = 0.133). Our results suggest that polymorphisms in *MTHFR* affect the susceptibility to gastric tumors in the Brazilian population and may be a factor causing poor prognosis in such patients.

Key words: Folate pathway; Gastric adenocarcinoma; Polymorphisms

INTRODUCTION

Although the incidence and mortality rates of gastric cancer (GC) have decreased worldwide over the past 25 years, this disease remains the 4th-most common cancer and the 2nd leading cause of death by cancer worldwide (Jemal et al., 2011). In South America, Brazil shows a relatively high GC incidence, particularly in Pará State, where the estimated incidence for 2012-2013 was 680 new cases (INCA, 2014).

In addition to well-known risk factors, such as *Helicobacter pylori* infection, several epidemiological studies have associated low folate levels with an increased risk of GC, because the folate metabolic pathway is involved in the synthesis and methylation of DNA (Götze et al., 2007).

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant form of folate in blood. MTHFR is also the methyl donor for the conversion of homocysteine to methionine (Stern et al., 2000a), the immediate precursor of *S*-adenosylmethionine, an important methyl donor in DNA methylation (Stern et al., 2000b). The most studied polymorphisms of the gene *MTHFR* include a C→T substitution at position 677 in exon 4 (*MTHFR* 677C>T), resulting in an amino acid substitution from valine to alanine (Ala222Val), which is correlated with enzyme activity and thermolability *in vitro*. Genotypes CT and TT had 65 and 30% of normal enzyme activity, respectively (Dong et al., 2008).

Thymidylate synthase (TS), another key enzyme in folate metabolism, is involved in the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate using 5,10-methylenetetrahydrofolate as a methyl donor (Sharp and Little, 2004). Therefore, this enzyme is related with dNTP balance and consequently with DNA synthesis and repair. TS expression levels were found to be associated with the response to 5-fluorouracil treatment (Pullarkat et al., 2001), a chemotherapy agent used to treat GC.

TS contains 3 functional polymorphisms in its 5'- and 3'-untranslated regions (UTRs). The first is a series of 28-base pair (bp) tandem repeats in the 5'-UTR of the thymidylate synthase enhancer region (TSER), resulting in 2 common alleles with double (2R) and triple (3R) repeats involved in the modulation of mRNA expression (Horie et al., 1995). The second is a single-nucleotide polymorphism (SNP) of C→G observed only in the 2nd repeat of the 3R allele (C/G SNP), creating 2 alleles, 3R (G)

and 3R (C), related to the transcriptional and translational activity of TS (Kawakami and Watanabe, 2003). The third is a 6-bp (TTAAAG) insertion at position 1494 in the 3'-UTR (1494del/ins6), which is associated with mRNA instability and translation (Ulrich et al., 2002).

In the present study, we investigated the association between *TS* and *MTHFR* polymorphisms and the susceptibility to GC in a population of Pará State, Northern Brazil.

MATERIAL AND METHODS

Samples and DNA extraction

Patients subjected to gastrectomy at the Ofir Loyola and João de Barros Barreto hospitals with no previous treatment were selected for the present study. From 2003 to 2007, 57 samples of tumoral gastric mucosa were obtained and stored in liquid nitrogen until DNA extraction. Histological classification was conducted according to Lauren (1965) by pathologists from the hospitals. For control purposes, 67 blood samples were obtained from Laboratório de Análises Clínicas from cancer-free individuals. All subjects signed the informed consent before inclusion in the study and all procedures were approved by the ethical committee of the involved hospitals.

Genomic DNA extraction was conducted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer recommendations.

MTHFR 677C>T genotyping

The 320-bp fragment of *MTHFR* containing the 677C>T polymorphism was amplified by polymerase chain reaction (PCR) using the following primers: forward 5'-AAGCAGAGGACTCTCTCTGCCC-3' and reverse 5'-CCCCCAGCCTGTGCGAGGACGGT-3', designed in our laboratory and with an annealing temperature of 57°C for 35 cycles. The fragments obtained were purified using the EZ-10 Spin Column PCR Product Purification kit (Bio Basic, Markham, ON, Canada) following manufacturer instructions and sequenced using an ABI3130 automatic sequencer (Life Technologies, Carlsbad, CA, USA). Sequences were aligned using the BioEdit v7.0.5 software (Hall, 1999).

TSER genotyping

The fragment with 28 bp in tandem (TSER) was amplified by PCR using the primers and conditions described by Ulrich et al. (2002). Polymorphic bands of 215 (2R) and 243 (3R) were confirmed after 14% polyacrylamide gel electrophoresis and silver staining. To analyze the C/G SNP, samples with 3R alleles were purified using the EZ-10 Spin Column PCR Product Purification kit (Bio Basic), followed by digestion with *Hae*III (Invitrogen, Carlsbad, CA, USA) and visualized by silver staining after 12% polyacrylamide gel electrophoresis.

1494del/ins6 genotyping

A fragment of 174 bp was distinguished by PCR using the following: primers forward 5'-TTCCCTCAAATCTGAGGGAGCTG-3' and reverse 5'-CTGCTCAGTTCCTAAAATA-3', designed in our lab, with an annealing temperature of 62°C for 35 cycles. The PCR products were purified

as above, followed by *Dra*I digestion (Invitrogen) and silver staining after 8% polyacrylamide gel electrophoresis. After digestion, the allele with the 6-bp insertion generated fragments of 109 and 65 bp, while the allele without the 6-bp insertion remained undigested.

Statistical analysis

Statistically significant differences were evaluated using the Chi-square test, G-test, and Fisher exact test. Odds ratios (ORs) with corresponding 95% confidence intervals (95%CIs) were used to measure the association between gastric cancer risk and the *MTHFR* and *TS* genotypes. In all tests, P values below 0.05 were considered to be statistically significant.

RESULTS

Among the cases, 38 (66.7%) were male and 19 (33.3%) were female, giving a male: female ratio of 2:1. In the control group, the same proportion was 1:1, with 32 (47.8%) males and 35 (52.2%) females. The average age of the patient group was 58 (± 11.9) years, ranging from 30 to 83 years, while that of the control group was 54.7 (± 12.5) years, ranging from 30 to 93 years.

For the TSER polymorphism, the frequency of the 2R allele was 0.48 for patients and 0.44 for the control group. No difference between cases and controls (P = 0.337) was observed. In the OR test, we observed an increased risk of GC in 2R/2R carriers (OR = 2.02, 95%CI = 0.78-5.22; P = 0.219) and a slightly increased risk of the development of tumors in the antrum (OR = 1.88, 95%CI = 0.57-6.2; P = 0.465), of advanced stage (OR = 2.57, 95%CI = 0.05-13.16; P = 0.419), and lymph node metastasis (OR = 3.25, 95%CI = 0.64-16.44; P = 0.252) in 2R allele carriers, although the differences were not statistically significant.

For the 1494del/ins6 polymorphism, the frequency of the ins6 allele was 0.54 and 0.52 for the patient and control groups, respectively. The genotype distribution did not differ between cases and controls (P = 0.937). We found that the presence of at least one ins6 allele increased the risk of developing advanced stage tumors (OR = 3.53, 95%CI = 0.40-30.55; P = 0.419) and with lymph node metastasis (OR = 4.37, 95%CI = 0.50-37.53; P = 0.287), but with no significant difference in the distributions among groups.

The combinations of all *TS* polymorphisms (TSER, C/G SNP, and 1494del/ins6) between cases and controls were analyzed to verify the relationship between polymorphisms in the 5' and 3' UTRs. The combination for this comparison followed that described by Yim et al. (2010), which considered both the 3R (G) and del6 alleles to be higher risk. No relationship between genotype distribution and an increased GC risk was observed.

For *MTHFR* C677T, the frequencies of the C allele were 0.74 and 0.75 for the patient and control groups, respectively.

Although the results were not statistically significant, we observed a modest increase in the risk of developing gastric tumors in samples with the TT genotype compared with the CC genotype (OR = 2.11, 95%CI = 0.49-9.15; P = 0.504) and CT genotype (OR = 3.18, 95%CI = 0.69-14.42; P = 0.239). When we correlated this polymorphism with histological type, tumor location, and lymph node metastasis, TT genotype carriers had a higher risk of developing tumors in the antrum (P = 0.19 *vs* CC and P = 0.02 *vs* CT) and in the intestine (OR = 4.18, 95%CI = 0.66-26.41; P = 0.252 *vs* CC and OR = 2.25, 95%CI = 0.32-15.75; P = 0.725 *vs* CT). Additionally, carriers of at least 1 T allele had an increased risk of lymph node metastasis (OR = 3.00, 95%CI = 0.88-10.12; P = 0.133).

DISCUSSION

It is known that epidemiological studies have associated low folate levels with an increased risk of GC, as the folate metabolic pathway is involved in the synthesis and methylation of DNA (Götze et al., 2007). Two enzymes are mainly involved on the folate pathway: MTHFR and TS, coded by *MTHFR* and TS genes, respectively. Several polymorphisms were described for both genes, being C677T (*MTHFR* gene), TSER, C/G SNP and 1494del/ins6 (*TS* gene) the moststudied (Dong et al., 2008; Ulrich et al., 2002). Considering the above mentioned, the present study investigated the possible relationship between these polymorphisms and GC risk in a population of Pará State, Northern Brazil.

The frequencies of the *TS* polymorphisms analyzed (TSER, C/G SNP and 1493 del/ins6) were similar to the results found by Graziano et al. (2004) in Caucasians. For such gene, in all analysis, we did not observe any relationship between genotype distribution and an increased risk of GC. Several studies have assessed the potential of these polymorphisms in the individual susceptibility to cancer, but the results are contradictory for gastric tumors (Ulrich et al., 2002; Zhang et al., 2005; Yim et al., 2010).

For *MTHFR* C677T polymorphism, the allele frequencies observed were similar to the values described by Neves Filho et al. (2010) in a population from northeastern Brazil. Previous studies have indicated that the T allele is associated with an increased risk of GC, particularly in Caucasian and Chinese populations. Although our results were not statistically significant, T allele carriers showed a moderately increased risk of developing GC compared to the control group (Graziano et al., 2006; Lacasaña-Navarro et al., 2006).

Individuals with the T allele have reduced MTHFR enzymatic activity, which in the presence of low levels of folate in the blood affect both methylation and DNA synthesis pathways. Thus, the T allele appears to contribute to the tumorigenic process in 2 ways: i) leading to hypomethylation, which activates oncogenes and latent transposons and inducing chromosomal instability (Frosst et al., 1995); and ii) the availability of thymidylate, causing a nucleotide imbalance and increasing the probability of uracil incorporation into DNA, resulting in breaks and errors in DNA repair (Choi and Mason, 2002).

However, when there is an adequate intake of folate, the T allele may not promote cancer risk because the level of methyl groups appears to be sufficient for the production of deoxynucleotides (Lievers et al., 2001). This unique gene/nutrient interaction may explain the inconsistent results in case-control studies of cancer risk in carriers of the *MTHFR* 677T allele. Thus, our results may be explained by the interaction of the gene and folate intake, which was not evaluated in our analysis.

Despite the small sample size, our results suggest that the presence of *MTHFR* polymorphisms is a factor in the poor prognosis of patients with GC.

Conflicts of interest

The authors declare no conflict of interest.

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