Association between the C3435T single-nucleotide polymorphism of *multidrug resistance 1* gene and risk of gastric cancer

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Abstract. The *multidrug resistance 1* (*MDR1*) gene encodes P-glycoprotein, which confers resistance to antineoplastic drugs, but also affects the kinetic disposition of certain drugs and carcinogens. The C3435T polymorphism of the *MDR1* gene may influence the transport and excretion of carcinogens, increasing the risk of cancer. The aim of this study was to evaluate the association between this polymorphism and the risk of gastric cancer (GC). Ninety-eight patients with non-cardia GC and 203 healthy subjects participated in the study. DNA was extracted from leukocytes and the *MDR1* polymorphism was analyzed using PCR-RFLP. Serology was performed by ELISA for the investigation of infection with *Helicobacter pylori*. No significant difference in the genotype (p=0.668) or allele (p=0.745) frequency of the C3435T polymorphism was observed between the GC and control groups. There was no association between the genotypes studied and the risk of GC in patients infected with *H. pylori* (p=0.662). Patient survival was not correlated with the genotypes studied (p=0.454). No correlation was observed between the C3435T polymorphism of the *MDR1* gene and GC risk or prognosis in the population studied.

Introduction

Gastric cancer (GC) is the fourth most common type of cancer in the world and the second leading cause of death due to cancer. In general, the incidence of GC is two to three times higher in developing countries and the disease is more frequent among men than women (1-4). Gastric carcinogenesis is a multifactorial process involving endogenous and exogenous factors that cause genetic mutations. GC comprises different stages, such as superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and, finally, carcinoma (5). In addition to infection with *Helicobacter pylori*, epidemiological studies have demonstrated the importance of environmental factors, such as consumption of salty, canned and smoked foods for the genesis of GC (6).

P-glycoprotein (P-gp) is encoded by the *multidrug resistance 1* (*MDR1*) gene and confers resistance to multiple antineoplastic agents. In addition, this protein affects the kinetic disposition of certain drugs and carcinogens (7). Various isoforms of P-gp are known, which are classified into classes I, II and III. Classes I and II are related to multidrug resistance, whereas class III is involved in the transport of phospholipids (8,9). Although first detected in tumor cells, P-gp is also expressed in normal tissues. In the gastrointestinal tract, P-gp is the first defense of the body against oral exposure to drugs and toxins (10). P-gp is a membrane-bound transporter that was first identified to be responsible for the development of multidrug resistance (7). This glycoprotein acts as an ATP-dependent efflux pump that exports drugs, reducing the intercellular concentrations of different chemotherapeutic agents. P-gp shows an expression gradient in gastrointestinal epithelial cells, with this protein being more expressed in the apical region. However, it is also found in hepatic canaliculi and in the proximal tubules of the kidneys, where it contributes to the excretion of biliary and urinary products, respectively (10-13).

There is growing evidence that changes in the function and/or expression of the *MDR1* gene contribute to the pathogenesis of inflammatory diseases of the gastrointestinal tract (14). Researchers have demonstrated that a polymorphism in the C3435T region of the *MDR1* gene influences the development of different cancers, since it is directly related to the transport of potentially carcinogenic substances (15-19). Although there are several reports on the pharmacological and enzymatic action of P-gp, studies investigating the association of the *MDR1* gene polymorphism and GC are scarce. Therefore, the aim of the present study was to characterize the genotypic profile of this genetic polymorphism and to correlate this polymorphism with the risk of GC and tumor aggressiveness.

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Abbreviations: CG, gastric cancer; P-gp, P-glycoprotein; SNP, single-nucleotide polymorphism; MDR1, multidrug resistance 1; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; dNTP, deoxynucleotide triphosphate

Key words: polymorphism, gastric cancer, multidrug resistance 1

Patients and methods

Sample population. A case-control study was conducted. The case group consisted of patients with GC seen at the outpatient clinic of the Discipline of Clinical Gastroenterology, Federal University of Sao Paulo-Escola Paulista de Medicina, Brazil. Patients with a confirmed histological diagnosis of non-cardia adenocarcinoma were invited to participate in the study. The control group consisted of healthy subjects who attended the blood collection service of the Central Laboratory of the Sao Paulo Hospital. The study was approved by the Ethics Committee of the institution, and all patients signed an informed consent form.

Assay methods. An ELISA kit (R-Biopharm GmbH, Germany) was used for the detection of serum anti-*H. pylori* IgG antibodies. DNA was extracted from peripheral venous blood leukocytes collected with EDTA as an anticoagulant using the Blood Spin Mini kit (Invisorb, Germany). The samples were submitted to polymerase chain reaction (PCR) and then genotyped using the PCR-restriction fragment length polymorphism (RFLP) technique. The following primers were used: forward 5'-TGC TGG TCC TGA AGT TGA TCT GTG AAC-3' and reverse 5'-ACA TTA GGC AGT GAC TCG ATG AAG GCA-3'. The reaction mixture for PCR contained 40 ng DNA, 1X reagent buffer, 0.125 mmol/l of each deoxynucleotide triphosphate (dNTP), 1.5 mmol/l $MgCl_2$, 0.75 μmol/l of each primer and 0.5 units Platinum *Taq* DNA polymerase in a final volume of 10 μl. DNA was denatured at 94˚C for 2 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, with a final extension at 72˚C for 4 min. After amplification, the products were digested with 5 units of the restriction enzyme *Mbo*I (New England Biolabs) at 37˚C for 3 h and the fragments were visualized on 3% agarose gel stained with ethidium bromide. Genome sequencing was used to confirm the PCR and RFLP techniques using random samples of the two groups. The PCR product of the *MDR1* gene was purified using the Big Dye XTerminator kit (Applied Biosystems) and sequenced in an ABI Prism 3100 sequencer (Applied Biosystems). The reverse primer was used for sequencing. The electropherogram was analyzed with the Sequence Scanner v1.0 program.

Statistical analyses. The Statistical Package for the Social Sciences v19.0 was used for statistical analysis. Age was compared between groups by the Student's t-test. The Chi-square test was used to determine differences in genotypes and alleles between the two groups. Survival was estimated by the Kaplan-Meier method and survival curves were compared by the log-rank test.

Results

The case group consisted of 98 patients with GC, including 43 (43.9%) women. The control group consisted of 203 subjects, including 110 (54.2%) women. The patients of the case and control groups were admitted during the same period. There was no difference in gender (p=0.12) or age (p=0.125) between the two groups (Table I).

Table I. Characteristics of the patients with gastric cancer and controls.

	Control n=203 (%)	Cancer n=98 (%)	p-value[a]
Gender			0.120
Female	110 (54.2)	43 (43.9)	
Male	93 (45.8)	55 (56.1)	
Age (years)			0.125
Mean ± SD	63.2±12.2	60.4±12.4	

[a]χ^2-test.

Specific primers were used for the amplification of a DNA fragment of the *MDR1* gene polymorphism, which resulted in a product of 248 bp. Digestion of the amplicon with *Mbo*I produced four bands of 232, 172, 60 and 16 bp in patients carrying the heterozygous CT genotype, two bands of 232 and 16 bp in patients with the homozygous mutant TT genotype and three bands of 232, 172 and 16 bp in patients carrying the homozygous CC genotype (Fig. 1).

In the control population, the genotypes were in Hardy-Weinberg equilibrium and the frequency of TT, CC and CT genotypes was 19.2, 33.0 and 47.8%, respectively. In patients with GC, the frequency of TT, CC and CT genotypes was 15.3, 32.7 and 52.0%, respectively. Genotype analysis showed no difference in genotype frequencies between patients with GC and controls (p=0.668). Similarly, no significant difference in the frequency of the C and T alleles was observed between patients with GC and healthy controls (p=0.745) (Table II). Comparison of genotypes between patients with metastatic (stage IV) and non-metastatic GC (stages I, II and III) showed no significant difference (p=0.257).

The number of subjects infected with *H. pylori* was similar in the two groups (GC: n=43, 43.9%; control: n=111, 54.7%) (p=0.160). No significant difference in CC, TT and CT genotype frequencies was observed between patients with GC (p=0.662) or controls (p=0.399) infected or not with *H. pylori* (Table III). There was no difference in survival between the genotypes studied (p=0.454) (Fig. 2).

Discussion

According to the Brazilian National Cancer Institute (INCA) (1), GC was the most frequently diagnosed cancer among men (64.3%) in 2010, with a higher incidence of the disease in men and women above the age of 50. A higher prevalence of GC among men (56.1%) was also observed in the present study. The mean age of the patients was 60.2 years. Studying cases of acute leukemia in India, Rao *et al* (20) observed a higher percentage of the TT genotype (51.7%) among patients with acute lymphoid leukemia (ALL) when compared to the control group (28.9%). However, no association was observed for patients with acute myeloid leukemia. In that study, the CC genotype was associated with a poorer prognosis of ALL. By contrast, Jamroziak *et al* (21) found a higher frequency of the CT genotype (48%) in Polish patients with ALL, but the differ-

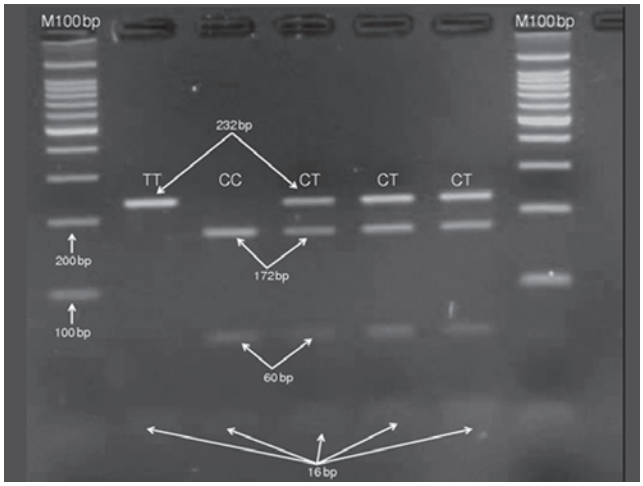

Figure 1. Detection of the length of polymorphisms by PCR followed by agarose gel electrophoresis. Wild-type CC genotype showing three fragments (172, 60 and 16 bp); homozygous mutant TT genotype showing two fragments (232 and 16 bp) and heterozygous CT genotype showing four fragments (232, 172, 60 and 16 bp).

ence was not significant. A higher frequency of the T allele and CT genotype has been observed in Iranian breast cancer patients when compared to a group without cancer, but the difference was not significant (18). The C3435T polymorphism of the *MDR1* gene has been investigated in inflammatory diseases, such as peptic ulcer, and in cancer, but the results are contradictory. In intestinal inflammatory disease, Schwab *et al* (22) found a higher frequency of the T allele and TT genotype of the *MDR1* gene in German patients with ulcerative colitis, but not in patients with Crohn's disease when compared to healthy subjects. Tahara *et al* (17) showed that the TT genotype of the *MDR1* gene was associated with a reduced risk of GC in the Japanese population. The authors also investigated the effect of the *MDR1* polymorphism on the risk of GC in patients infected or not with *H. pylori* and found that patients carrying the TT genotype had a lower risk of the disease, irrespective of the presence or absence of *H. pylori* infection (17). Also in Japan, Sugimoto *et al* (16) demonstrated a higher incidence of the CT genotype in patients with GC, gastric and duodenal ulcer and gastritis, but the difference was not significant when compared to the control group. The homozygous TT genotype was found to be associated with a higher risk of GC in an Iranian population when compared to a control group (23). Comparison of the frequencies of the C and T alleles between patients with GC and controls showed a higher prevalence of the C allele in the two groups (58.7 and 56.9%, respectively), but the difference was not significant. CT genotype was the most frequent in both the control group and the case group (47.2 and 52%, respectively), but the finding was again not statistically significant. Balram *et al* (24) compared the frequency of the C allele among Asian, European and African populations and found a mean frequency of 43% in the Asian population, 52% in European Caucasians and 78% in Africans. In the present study, the frequency of the C allele was 56.9% in the control group and 58.7% in patients with GC, values similar to those reported for European Caucasians.

Gastric carcinogenesis is a multifactorial process and infection with *H. pylori* is one of the risk factors (5,6). In

Table II. Genotype and allele frequency in patients with gastric cancer and controls.

	Control n=203 (%)	Cancer n=98 (%)	p-value[a]
Genotype			0.668
TT	39 (19.2)	15 (15.3)	
CC	67 (33.0)	32 (32.7)	
CT	97 (47.8)	51 (52.0)	
Allele			0.745
T	175 (43.1)	81 (41.3)	
C	231 (56.9)	115 (58.7)	

[a]Fisher's exact test and χ^2-test.

Table III. Frequency of the *MDR1* polymorphism in patients with gastric cancer and controls infected or not with *Helicobacter pylori*.

	CC n (%)	TT n (%)	CT n (%)	p-value[a]
Cancer				0.662
H. pylori-positive	5 (5.1)	15 (15.3)	23 (23.5)	
H. pylori-negative	10 (10.2)	17 (17.2)	28 (28.6)	
Control				0.399
H. pylori-positive	21 (10.3)	41 (20.2)	49 (24.1)	
H. pylori-negative	18 (8.9)	26 (12.8)	48 (23.6)	

[a]χ^2-test.

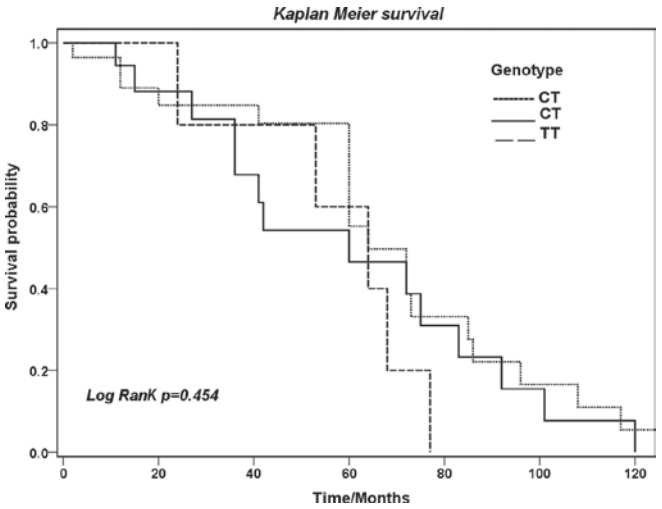

Figure 2. Kaplan-Meier curves of the presence of the C3435T polymorphism in overall survival (p=0.454, log-rank test) among GC patients, where the C/T, T/T and C/C genotypes are combined. Log-rank test was based on the full data of GC patients.

Japan, Sugimoto *et al* (17) found no association between the *MDR1* C3435T polymorphism and the risk of GC in patients infected with *H. pylori* or in patients with peptic ulcer. In

the present study, we also observed no difference between genotypes, infection with *H. pylori* and GC risk. The results suggest that *H. pylori* infection is not associated with the *MDR1* C3435T polymorphism. Patients with advanced-stage GC had poor survival (p=0.003). However, no difference in survival was observed between the genotypes studied. Although *MDR1* T-55C and BCRP C43A were reported to be functional SNPs that change their protein functions (25,26), the C3435T SNPs analyzed in this study had no association with overall survival. This suggests that this gene is probably not the most important determinant in survival analysis. However, it is also possible that the limited number of SNPs selected for this study missed the most important functional variants of this gene. Further investigation is required to illustrate how these genes may affect the clinical outcome of GC. The differences in the results of studies investigating the *MDR1* C3435T polymorphism may be related to differences in the ethnic background of the populations studied and in the number of patients included in each study. In addition, the *MDR1* C3435T SNP is located in a non-coding region and therefore may not interfere with the expression of P-gp (23), a fact explaining the lack of association between this polymorphism and gastric carcinogenesis.

In conclusion, the present results provide evidence that the C3435T polymorphism of the *MDR1* gene is not associated with susceptibility to gastric cancer. No correlation was observed between this polymorphism and infection with *H. pylori* or survival.

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