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ORIGINAL RESEARCH

Susceptibility of Genetic Variations in Methylation Pathway to Gastric Cancer

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Background: DNA methylation in the CpG island is associated with gastric cancer, genetic variations residue in genes involved in methylation pathway could contribute to the occurrence of gastric cancer. Here, we investigated the association between *DNMTs* (*DNMT1/DNMT3A/DNMT3B*), *MTHFR* genetic variations and gastric cancer risk and patients' survival.

Patients and Methods: We recruited 490 gastric cancer patients and 488 age- and sex-matched healthy controls. The genotypes of the genetic variations were detected by a Mass-array platform. A commercial *Helicobacter pylori* (*H. pylori*) immunogold testing kit was used to determine the *H. pylori* infection.

Results: We found that carriers of *DNMT1* rs2228612C allele was associated with decreased gastric cancer risk (CT vs. TT: adjusted OR = 0.70, 95% CI = 0.53–0.94, P = 0.02; CT/CC vs.TT: adjusted OR = 0.73, 95% CI = 0.56–0.96, P = 0.02). Further stratified analysis showed that *DNMT1* rs2228612 CT/CC were associated with a decreased gastric cancer risk in the subgroups of age ≤64 years old (adjusted OR = 0.61, 95% CI = 0.41–0.90, P = 0.01), male (adjusted OR = 0.72, 95% CI = 0.53–0.98, P = 0.03), negative *H. pylori* infection (adjusted OR = 0.67, 95% CI = 0.45–0.98, P = 0.04), tumor stage T3-T4 (adjusted OR = 0.69, 95% CI = 0.51–0.92, P = 0.01), and non-gastric cardiac adenocarcinoma (NGCA) (adjusted OR = 0.72, 95% CI = 0.54–0.97, P = 0.03). However, none of the genetic variations of this study was associated with overall survival.

Conclusion: We concluded that the *DNMT1* rs2228612C genotype is a protective factor for gastric cancer in Han Chinese population.

Keywords: DNMTs, MTHFR, genetic variation, gastric cancer

Introduction

Gastric cancer is one of the most prevalent cancers in the world, ranking fifth among the most common cancers and third among cancer-related deaths, *Helicobacter pylori* (*H. pylori*) infection, age, living habits and diets (such as high salt intake, low fruit and vegetables), are proved as risk factors for gastric cancer.[1] Specifically, *H. pylori* colonization in the stomach could result in chronic gastritis and may result in gastric cancer eventually. Therefore, clearance of *H. pylori* could reduce the risk of gastric cancer.[2] In recent years, despite the decrease in global gastric cancer incidence, the incidence in East Asia is still high, especially in China.[3] Therefore, to ascertain the risk of gastric cancer is of great significance.

Dysregulated gene expression in cancer caused by DNA methylation has been reported widely. Three main types of DNA methyltransferase (DNMTs: DNMT1, DNMT2 and DNMT3) are related with genomic methylation. For example, by activating the NF-κB pathway and regulating DNMT3b, *H. pylori* silenced NDRG2 (N-myc downstream-regulated gene 2) then promoting gastric cancer progression.[4] Similarly, NDRG1 was down-regulated in gastric cancer by promoter DNA methylation.[5] Methylation at CpG islands is a critical mechanism of gene silencing in gastric cancer.[6] Besides, DNMT1 was

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reported to maintain these methylation patterns in the period of DNA replication. Act as *de novo* methyltransferases, DNMT3A and DNMT3B were reported to establish methylation patterns during embryogenesis.[7] Several studies also indicated that upregulation of *DNMTs* can promote tumor progression, invasion and metastasis through down regulation of genes that play a role in proliferation inhibition and apoptosis-related pathway.[8] Additionally, genetic variations in DNA methyltransferases, *DNMT1, DNMT3A, DNMT3B* were suggested to be associated with oral squamous cell carcinomas.[9] In DNA methylation pathway, folate metabolism involves in DNA methylation, repair and synthesis, and methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme involved in the folate pathway.[10] Increasing studies discovered that genetic variations in *MTHFR* may affect the enzymatic activity of the encoding protein and contribute to the development of cancers. *MTHFR* C677T (rs1801133) variations was also investigated for gastric cancer risk, but the results were inconsistent.[11] Interestingly, studies have shown that *MTHFR* C677T polymorphism is associated with increased risk of gastric cancer and decreased risk of cardia gastric cancer in Chinese population.[12]

Based on the genetic variations in DNA methylation pathway involved in occurence of gastric cancer, here we conducted a case–control study on genotyped SNPs in a Chinese population to assess the association between variants in 4 genes (*DNMT1, DNMT3A, DNMT3B* and *MTHFR*) and susceptibility to gastric cancer. Nine genetic variations (*DNMT1*: rs16999593, rs10420321, rs2228612, rs7560488; *DNMT3A*: rs13420827, rs1550117; *DNMT3B*: rs1569686), in *DNMTs* and *MTHFR* (rs1476413, rs1801131) were selected to evaluate their susceptibility to the risk of gastric cancer, as well as their survival predictor role in gastric cancer patients.

Materials and Methods

Study Subjects

A total of 490 gastric cancer patients and 488 age- and sex-matched healthy controls were recruited in this study.[13] All patients were histologically diagnosed with gastric cancer, the controls were individuals who came to the hospital for routine physical examination. The patients and healthy controls information were collected from the hospital records and questionnaire respectively. The clinical stages of gastric cancer were classified according to the 6th edition of the American Joint Commission for Cancer Staging Manual. The survival status of gastric cancer patients were obtained by on-site interviews, direct calling, or reviews of medical charts. The Institutional Review Board of the Nanjing First Hospital approved the study protocol, and written informed consent was obtained from all of the participants.

DNA Extraction and Genotyping

According to the manufacture's protocol, the patient's blood samples were collected to extract DNA by using GoldMag-Mini Whole Blood Genomic DNA Purification Kit (GoldMag Co. Ltd. Xi'an, China). The purity of the collected DNA was determined by spectroscopy (DU530UV/VIS spectrophotometer, Beckman Instruments, Fullerton, CA, USA). The Sequenom Mass-array platform was used to genotype all samples. SequenomTyper 4.0 Software was used for the data analysis.

We selected the *DNMT1/DNMT3A/DNMT3B* and *MTHFR* genetic variations to evaluate their associations with gastric cancer. For selecting the genetic variations, we retrieved the information from the National Center for Biotechnology Information dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP). Then, the following criteria were built for selecting the genetic variations: (1) the minor allele frequency (MAF) was ≥ 5% in the Han Chinese population; (2) the variation was located in an exon, promoter region (less than 2 kb apart from the transcription start), 5′untranslated region (UTR), or 3′UTR; (3) the genetic variation has been reported that correlated with cancer risk. Finally, nine *DNMT1/DNMT3A/DNMT3B* genetic variations and two *MTHFR* genetic variations were selected to study further (Table S1).

H. pylori Assay on Serum

Commercial *H. pylori* immuno-gold testing kit (Kangmei Tianhong Biotech Co., Ltd, Beijing, China) was used to detect *H. pylori* antibodies in the sera of the participants. The sensitivity and specificity of the kit were 98.3% and 98.5% respectively.

Statistical Analysis

The difference of population characteristics between the case and control group was calculated by Chi square test (χ^2) or *t* test, and the Hardy-Weinberg equilibrium (HWE) balance of the control group was calculated by Chi-square test of goodness of fit. In order to test the relationship between genetic variations and cancer, logistic regression of SAS software (Version 9.1; SAS Institute, Cary, NC, USA) was used to calculate odds ratios (ORs) and 95% confidence interval (CIs). Clinical and pathological characteristics subgroup analysis of cancer were used to prove whether genetic variations were still associated with cancer risk in subgroup. For patients with wild-type gene compared with other genes, the survival of cancer patients were used to calculate the hazard ratios (HR) and 95% confidence interval. The calculation method was the Cox regression model of SPSS (SPSS, Chicago, IL, USA) by using the log-rank test. A two-sided *P* value < 0.05 was considered statistically significant.

Results

Characteristics of the Study Population

The results of HWE analysis showed that the genotype results of nine genetic variations conformed to follow HWE ($P > 0.05$) (Table S1). The demographic and exposure data of all the participants are summarized in Table S2. There were no differences between the two groups for age ($P = 0.748$), gender ($P = 0.916$) and *H. pylori* infection ($P = 0.055$). The frequencies of cigarette smoking and alcohol consumption in the patients were higher than those in the controls. The distributions of the genetic variations in patients and the controls were showed in Table 1.

Associations Between Genetic Variations and Gastric Cancer Risk

There was a significant difference in the distribution of the *DNMT1* rs2228612 genotype between the case group and the control group. The result showed that the *DNMT1* rs2228612CT (CT vs.TT: adjusted OR = 0.70, 95% CI = 0.53–0.94, $P = 0.02$) and CT/CC genotypes (CT/CC vs. TT: adjusted OR = 0.73, 95% CI = 0.56–0.96, $P = 0.02$) were associated with decreased gastric cancer risk, respectively. No significant association was observed between the other genetic variations and gastric cancer risk (Table 1).

To further assess the association between *DNMT1* rs2228612 and the risk of gastric cancer, we performed a stratified-analysis by age, gender, *H. pylori* infection status, tumor stage, and tumor site using a co-dominant model (CT/CC vs. TT). The decreased risk of *DNMT1* rs2228612C allele carriers (CT/CC) for gastric cancer remained significant in the following subgroups: age≤64 years old (adjusted OR = 0.61, 95% CI = 0.41–0.90, $P = 0.01$), male (adjusted OR = 0.72, 95% CI = 0.53–0.98, $P = 0.03$), negative for *H. pylori* infection (adjusted OR = 0.67, 95% CI = 0.45–0.98, $P = 0.04$), tumor stage T3-T4 (adjusted OR = 0.69, 95% CI = 0.51–0.92, $P = 0.01$), non-gastric cardiac adenocarcinoma (NGCA; adjusted OR = 0.72, 95% CI = 0.54–0.97, $P = 0.03$, Table 2).

Association Between Genetic Variations and Clinical Outcomes

In order to assess the relationship between patient survival and genetic variations, a total of 477 patients were followed up to five years for the overall survival (OS), and a Cox regression analysis is used to calculate HRs for patients to evaluate the predictive value of the genetic variations to patients' survival. The comparison of wild type with those who with any mutantant allele revealed that no association between the genetic variations and OS (Table 3), indicating that these genetic variations have no predictive value for gastric cancer patients' survival.

Discussion

In this population-based study, 490 gastric cancer patients and 488 age- and gender-matched healthy controls in a Chinese population were recruited. The result showed that the *DNMT1* rs2228612C allele was related with decreased risk of gastric cancer and that such an association was maintained in the subgroups of age≤64 years old, male, negative for *H. pylori* infection, tumor stage T3-T4, non-gastric cardiac adenocarcinoma (NGCA). Whereas, all the enrolled nine genetic variations were not associated with gastric cancer patients' survival.

Table 1 Associations Between *DNMT1/DNMT3A/DNMT3B* and *MTHFR* genetic variations and Gastric Cancer Risk

Genotype	Cases, n(%)	Controls, n(%)	OR (95% CI)	AOR (95% CI)[a]	p-value
DNMT1 rs16999593					
TT	324(66.12)	318(65.16)	Reference	Reference	
CT	148(30.20)	148(30.33)	0.98(0.75–1.29)	0.93(0.70–1.23)	0.61
CC	18 (3.67)	22(4.51)	0.80(0.42–1.53)	0.84(0.44–1.60)	0.59
CT/CC	166(33.87)	170(34.84)	0.96(0.74–1.25)	0.92(0.70–1.20)	0.53
DNMT1 rs10420321					
AA	167(34.08)	161(32.99)	Reference	Reference	
AG	223(45.51)	221(45.29)	0.97(0.73–1.29)	0.94(0.70–1.26)	0.69
GG	100(20.41)	106(21.72)	0.91(0.64–1.29)	0.89(0.62–1.27)	0.52
AG/GG	323(65.92)	327(67.01)	0.95(0.73–1.24)	0.93(0.71–1.21)	0.58
DNMT1 rs2228612					
TT	199(40.7)	165(33.81)	Reference	Reference	
CT	196(40.08)	225(46.11)	0.72(0.55–0.96)	0.70(0.53–0.94)	0.02
CC	94(19.22)	98(20.08)	0.80(0.56–1.13)	0.80(0.56–1.14)	0.21
CT/CC	290(59.30)	323(66.19)	0.74(0.57–0.97)	0.73(0.56–0.96)	0.02
DNMT1 rs7560488					
TT	328(66.94)	319(65.37)	Reference	Reference	
TC	148(30.20)	152(31.15)	0.95(0.72–1.25)	0.98(0.74–1.29)	0.86
CC	14(2.86)	17(3.48)	0.80(0.39–1.65)	0.78 (0.37–1.62)	0.50
TC/CC	162(33.06)	169(34.63)	0.93(0.72–1.22)	0.95(0.73–1.25)	0.72
DNMT3A rs13420827					
CC	323(65.92)	307(62.91)	Reference	Reference	
GC	150(30.61)	163(33.40)	0.88(0.67–1.15)	0.87(0.66–1.15)	0.34
GG	17(3.47)	18(3.69)	0.90(0.45–1.77)	0.88(0.44–1.75)	0.71
GC/GG	167(34.08)	181(37.09)	0.88(0.68–1.14)	0.87(0.67–1.13)	0.30
DNMT3A rs1550117					
GG	321(65.51)	305(62.50)	Reference	Reference	
AG	152(31.02)	164(33.61)	0.88(0.67–1.15)	0.87(0.66–1.15)	0.33
AA	17(3.47)	19(3.89)	0.85(0.43–1.67)	0.85(0.43–1.67)	0.63
AG/AA	169(34.49)	183(37.5)	0.88(0.68–1.14)	0.87(0.67–1.13)	0.29
DNMT3B rs1569686					
TT	423(86.33)	416(85.25)	Reference	Reference	
GT	61(12.45)	70(14.34)	0.86(0.59–1.24)	0.85(0.58–1.24)	0.40
GG	6(1.22)	2(0.41)	2.95(0.59–14.70)	3.33(0.66–16.73)	0.14
GT/GG	67(13.67)	72(14.75)	0.92(0.64–1.31)	0.92(0.64–1.32)	0.64
MTHFR rs1476413					
CC	324(66.12)	329(67.42)	Reference	Reference	
CT	152(31.02)	146(29.92)	1.06(0.80–1.39)	1.03(0.78–1.37)	0.81
TT	14(2.86)	13(2.66)	1.09(0.51–2.36)	1.17(0.53–2.56)	0.70
CT/TT	166(33.88)	159(32.58)	1.06(0.81–1.38)	1.05(0.80–1.37)	0.74
MTHFR rs1801131					
TT	327(66.73)	333(68.24)	Reference	Reference	
GT	149(30.41)	142(29.10)	1.07(0.81–1.41)	1.05(0.79–1.39)	0.75
GG	14(2.86)	13(2.66)	1.10(0.51–2.37)	1.17(0.54–2.57)	0.69
GT/GG	163(33.27)	155(31.76)	1.07(0.82–1.40)	1.06(0.81–1.39)	0.68

Note: [a]Adjusted by age, smoking, drinking, and *H. pylori* infection status.
Abbreviation: AOR, adjusted OR.

Previously, *DNMT1* genetic variations has been reported to be associated with various diseases, such as autosomal dominant cerebellar ataxia-deafness and narcolepsy, hereditary sensory neuropathy with dementia and hearing loss.[14] In addition, many studies have shown that DNMT1 is involved in the occurrence and development of tumors. Mechanically,

Table 2 Subgroup Analysis of rs2228612 to Gastric Cancer Risk

Variables	rs2228612 (Cases/Controls)		AOR (95% CI)[a]	p-value
	TT	CT/CC		
Age				
≤64	99/73	131/154	0.61(0.41–0.90)	0.01
>64	100/92	159/169	0.87(0.61–1.25)	0.46
Gender				
Male	148/121	210/237	0.72(0.53–0.98)	0.03
Female	51/44	80/86	0.79(0.47–1.32)	0.37
H. pylori infection				
Positive	111/86	157/152	0.81(0.56–1.17)	0.26
Negative	88/79	133/171	0.67(0.45–0.98)	0.04
Differentiation				
Low	109/165	161/323	0.73(0.54–1.00)	0.05
Median to high	84/165	124/323	0.74(0.53–1.05)	0.09
Clinical stage				
I–II	61/165	96/323	0.84(0.57–1.24)	0.38
III–IV	138/165	194/323	0.69(0.51–0.92)	0.01
Tumor site				
Cardia	56/165	84/323	0.75(0.50–1.10)	0.14
Non-cardia	143/165	206/323	0.72(0.54–0.97)	0.03

Note: [a]Adjusted by age, smoking, drinking, and *H. pylori* infection status.
Abbreviation: AOR, adjusted OR.

knockdown of *DNMT1* dysregulates tumor-suppressor P21 and the apoptosis inducer BIK (Bcl-2 interacting killer),[15] and inhibits crosstalk of *DNMT1* and oestrogen receptor-related receptor alpha (ERRα), resulting in breast cancer progression by regulating the expression of IRF4 (Interferon Regulatory Factor-4).[16] Moreover, as a mediator, *DNMT1* could promote carcinogenesis and progression of gastric cancer via various regulate networks.[17–21] Meanwhile, the expression of *DNMT1* could served as a survival biomarker for gastric cancer patients for that down regulation of *DNMT1* could increase cisplatin sensitivity and high expression of *DNMT1* predicted poor gastric cancer patients' survival.[22] Here, we observed that a genetic variation in *DNMT1* (rs2228612) was susceptible to risk of gastric cancer. Actually, in recent years, several studies have shown that *DNMT1* rs2228612G/A genotype was associated with decreased risk of breast cancer,[23] which was consistent with our result. Whereas, an increased risk of *DNMT1* rs2228612 GG genotype for breast cancer risk was also reported in a Chinese Guangdong population. For gastric cancer, three studies invested the *DNMT1* rs2228612 in a Chinese population that, one reported no significant association, but they reported *DNMT1* rs2228612 GG genotype acted as a protective factor for esophageal cancer, which was consistent with our results.[24] Unfortunately, one study omitted the data due to fail to follow HWE,[25] and another study reported it was not associated with gastric cancer patients' survival,[26] yet which was consistent with our result. Moreover, in this study, they reported the MAF (C/G allele) in *DNMT1* rs2228612 was 0.431 in controls, which was consistent with the result (MAF=0.388) in Asian population of dbSNP database, and the study reported in a China population (MAF=0.450).[27] The genotyping of this study was based on the Mass-array platform, which was reliable for genetic variation detection. Actually, due to limited published data regarding *DNMT1* rs2228612 and gastric cancer, our result should be confirmed by further study with larger sample size .

DNMT1 rs2228611 is a synonymous genetic variation locates in exon 17, while *DNMT1* rs2228612 locates in exon 12, whereas, these two genetic variations were not in linkage disequilibrium each other, according to previous report in a Chinese population.[28] Studies have shown that substitution of phenylalanine by isoleucine at 327 amino acid in DNMT1 caused by *DNMT1* rs2228612 (A/G) may affect the function of DNMT1 and involve in the carcinogenesis by regulating gene expression through effecting the CpG island hypermethylation statue. Additionally, we also found the significant association between *DNMT1* rs2228612 and decreased risk of gastric cancer was maintained in the subgroup

Table 3 Analysis of Associations Between Genetic Variations and Clinical Outcomes

Genotype	Cases, n	Death, n (%)	Log-Rank *p*-value	HR
rs16999593				
TT	313	207 (66.1)		Reference
CT/CC	164	108 (65.9)	0.923	0.989 (0.783–1.248)
rs10420321				
AA	160	104 (65)		Reference
AG/GG	317	211 (66.6)	0.950	1.007 (0.797–1.274)
rs2228612				
TT	191	121 (63.4)		Reference
CT/CC	285	194 (68.1)	0.587	1.065 (0.849–1.336)
rs7560488				
TT	321	210 (65.4)		Reference
CT/CC	156	105 (67.3)	0.514	1.081 (0.855–1.367)
rs13420827				
CC	314	206 (65.6)		Reference
GC/GG	163	109 (66.9)	0.654	1.054 (0.836–1.330)
rs1550117				
GG	313	200 (63.9)		Reference
AG/AA	164	115 (70.1)	0.392	1.105 (0.879–1.391)
rs1569686				
TT	411	271 (65.9)		Reference
GT/GG	66	44 (66.7)	0.947	1.011 (0.735–1.390)
rs1476413				
CC	318	216 (67.9)		Reference
CT/TT	159	99 (62.3)	0.105	0.821 (0.647–1.042)
rs1801131				
TT	321	219 (68.2)		Reference
GT/GG	156	96 (61.6)	0.064	0.797 (0.627–1.013)

of those who with age ≤ 64 years old, male, tumor stage T3-T4, non-gastric cardiac adenocarcinoma or negative for *H. pylori* infection, which may be attribute to the fact that younger patients are less likely to be exposed to risk factors, that male are more likely to smoke and drink than female, and that the incidence of gastric cancer with negative *H. pylori* infection is lower, respectively.

Although *DNMT1* rs2228612 was reported as an independent predictor of poor OS in melanoma patients;[29] however, we found none of the genetic variations are associated with the prognosis of gastric cancer, which was partly consistent to the previous study reported.[26] Admittedly, there is limitation in this study. The included subjects are from a single-centre, which may affect the study representation.

Conclusion

We concluded that *DNMT1* rs2228612C allele may play a protective role in gastric cancer in Han Chinese population. On the other hand, nine genetic variations (*DNMT1*: rs16999593, rs10420321, rs2228612, rs7560488; *DNMT3A*: rs13420827, rs1550117; *DNMT3B*: rs1569686) in *DNMTs* and *MTHFR* (rs1476413, rs1801131) was not found to associate with the survival of gastric cancer patients.

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Author Contributions

The work presented here was carried out in collaboration among all authors. All authors made a signifcant contribution to the study, including the conception, study design, execution, acquisition of data, analysis and interpretation. Also, all authors took part in drafting, revising and critically reviewing the manuscript. All authors gave final approval of the version to be published, agreed on the journal to which the article has been submitted, and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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