RESEARCH ARTICLE

DNMT3a rs1550117 Polymorphism Association with Increased Risk of *Helicobacter pylori* Infection

Xue-Yuan Cao[1], Zhi-Fang Jia[2], Dong-Hui Cao[2], Fei Kong[2], Mei-Shan Jin[3], Jian Suo[1], Jing Jiang[2]*

Abstract

Background: DNA methyltransferase-3a (*DNMT3a*) plays significant roles in embryogenesis and the generation of aberrant methylation in carcinogenesis. This study aimed to investigate associations between single nucleotide polymorphisms (SNPs) of the *DNMT3a* gene and risk of *Helicobacter pylori* infection, gastric atrophy and gastric cancer. Methods: The subjects comprised 447 patients with gastric cancer; 111 individuals with gastric atrophy and 961 healthy controls. Two SNPs (rs1550117 and rs13420827) of the *DNMT3a* gene were genotyped by Taqman assay. *DNMT3a* expression was analyzed in cancer tissues from 89 patients by tissue microarray technique. Odds ratio (ORs) and 95% confidence intervals were calculated by multivariate logistic regression. Results: Among healthy controls, risk of *H.pylori* infection was significantly higher in subjects with the rs1550117 AA genotype, compared to those with GG/AG genotypes of *DNMT3a* [OR=2.08, (95%CI: 1.02-4.32)]. However, no significant correlation was found between the two SNPs and risk of developing gastric atrophy or gastric cancer. In addition, no increase in *DNMT3a* expression was observed in the gastric cancer with *H.pylori* infection. Conclusions: This study revealed that *DNMT3a* rs1550117 polymorphism is significantly associated with an increased risk of *H. pylori* infection, but did not support any evidence for contributions of *DNMT3a* rs1550117 and rs13420827 to either gastric atrophy or gastric cancer. The biological roles of *DNMT3a* polymorphisms require further investigation.

Keywords: Polymorphisms - DNA methyltransferase 3a - *Helicobacter pylori* - gastric atrophy - gastric cancer

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Introduction

Gastric cancer (GC) is a major health problem. In 2011, 989,600 new cases were predicted and more than 738,000 deaths worldwide. Half of these cases and deaths were estimated to occur in China (Jemal et al., 2011; Bray et al., 2012). Helicobacter pylori (*H.pylori*) infection is the main cause of gastric atrophy (GA), gastroduodenal ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Lehours et al., 2003; Chuah et al., 2011). *H.pylori* infection was found in half of the population of the world, and the risk for non-cardiac GC in *H.pylori* infected person was estimated to be 3-20 fold or even higher (Kamangar et al., 2006; Malfertheiner et al., 2012). However, not all infected subjects eventually develop GA and only a small numbers develop to GC. Factors that influence the risks for atrophy and cancer in the presence of infection may be related to the characteristics of the host. Various studies show that inherited cancer susceptibility genes and SNPs are correlated with individual's risk of developing GC (Saeki et al., 2013).

Changes in epigenetic marks such as DNA methylation is associated with several diseases, including cancer, metabolic disorders, and various reproductive conditions (Cortessis et al., 2012). CpG island hypermethylation and genomic DNA hypomethylation are found in gastric tumors and premalignant lesions (Park et al., 2009), and *H.pylori* infection can induces aberrant CpG island hypermethylation in gastric mucosa (Nakajima et al., 2009; Park et al., 2009). Several reports indicated that accumulation of aberrant methylation in gastric mucosa produces a field for cancerization, and methylation levels correlate with gastric cancer risk (Guo et al., 2011; Wang et al., 2012). However, few studies have investigated the host factors, such as SNPs of DNA methyltransferases (DNMTs) gene, which may affect epigenetic regulation in gastric mucosa (Fan et al., 2010; Wu et al., 2012; Yang et al., 2012).

In our previous study among Chinese, subjects with rs10420321 GG and rs8111085 CC genotype of the DNMT1 gene were associated with reduced risks for *H.pylori* infection and higher risks for GA, supporting the hypothesis that polymorphisms of DNMTs may

*[1]Department of Gastric and Colorectal Surgery, [2]Division of Clinical Epidemiology, [3]Division of Pathology, First Hospital of Jilin University, Changchun, China *For correspondence: jiangjing19702000@jlu.edu.cn*

influence the enzyme activities to affect the *H.pylori* infection and carcinogenesis (Jiang et al., 2012). DNA methyltransferase-3a (*DNMT3a*) also plays a significant part in the development of embryogenesis and in the generation of aberrant methylation in carcinogenesis. The *DNMT3a* gene is located on chromosome 2p23 with a total size of 109.6 kb and is constituted of 26 exons (Weisenberger et al., 2002). Mutations in coding regions of the *DNMT3a* have been reported in various malignant hematological disorders, such as a missense mutation in codon 882 leading to an amino acid exchange (Kim et al., 2013). Also, allelic loss of *DNMT3a* has been frequently observed in many common solid cancers, including GC, lymphomas and lung cancers (Kim et al., 2013). Several studies have found that *DNMT3a* is over-expressed in human cancers, suggesting that it may be involved in tumorgenesis and tumor progression (Ding et al., 2008; He et al., 2011; Yang et al., 2011). Recently, *DNMT3a* SNPs rs1550117 and rs13420827 were also found to be significantly associated with GC (Fan et al., 2010; Wu et al., 2012; Yang et al., 2012). The aim of this study is to confirm the formerly reported association between the *DNMT3a* polymorphisms and GC in a large number of Chinese subjects, as well as to examine the association with *H.pylori* infection and GA.

Materials and Methods

Study populations

Four hundred and forty seven GC (non-cardiac GC) cases were selected from the First Hospital of Jilin University, between 2008 and 2010. All patients underwent tumor resections with histological confirmed gastric adenocarcinoma. Individuals with GA and healthy controls were recruited from health examinees in the health check-up centre of the same hospital from 2009 to 2010. In brief, a total of 1111 individuals without cancer history (654 males and 457 females, ages of 35 to 80 years old) participated in the study. The examinees were Han descent from the area of Changchun. 150 subjects were found to have GA by serum PG examination and 111 of them were confirmed by biopsy and histopathological examinations; 39 subjects were excluded from the study as they were either rejected endoscopic examination (22 cases) or diagnosed negative for GA by endoscopic examination and pathological examinations (17 cases). The remaining health examinees (961) were included in the control group. The inclusion criteria for the control group were: negative by serum PG screening. Subjects with a history of gastric disease, ulcer, and history of cancers were not considered candidates for the control. Informed consent was obtained from all patients and the study protocol was approved by the ethics committee of the First Hospital of Jilin University.

Tests for H.pylori infection and diagnosis of GA

Serum immunoglobulin (Ig) G antibodies to *H.pylori* were detected using a kit for *H.pylori* -Ig G enzyme-linked immunosorbent assay (ELISA) (Biohit, Helsink, Finland). The antibody titers were quantified by optical density (OD) readings according to the manufacturer's protocol and titres higher than the threshold value of 30EIU were considered as positive for *H.pylori* infection. Levels of PepsinogenI(PG I) and II in serum were measured using ELISA kits (Biohit, Helsink, Finland). For gastric atrophy screening, cut-off points used in this study were < 82.3 ng/ml for PG I and <6.05 for PGI/PG II ratio, as they had previously been validated against histological confirmatory studies for GA in Chinese population (Cao et al., 2007). The quality control samples in kits showed coefficients of variation of 4.5%, 4.3% and 4.7% for *H.pylori*, PG I and PG II, respectively. 111subject of GA suspected cases, based on the serum screening, were confirmed by endoscopy, biopsy and histological examinations for final diagnosis.

Genotyping

Genomic DNA from whole blood sample was extracted with an AxyPrep blood Genomic DNA extraction Kit (AP-MN-BL-GDNA-250, Axygen Biosciences, Union City, USA). Polymerase chain reactions (PCR) were carried out in a 5 ul per reaction on the genomic DNA (10 ng) using a TaqMan universal PCR master mix (Applied Biosystems). Forward, reverse primers, FAM and VIC labeled probes were designed by Applied Biosystems (ABI Assay-by-Designs). Sequences of primers and probes are available on request. Amplification conditions on BIO-RAD S1000TM thermal cyclers (Bio-Rad Laboratories, Hercules, California) were as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The PCR products were genotyped on an ABI PRISM 7900HT Sequence Detector in end-point mode using the Allelic Discrimination Sequence Detector Software V2.3 (Applied Biosystems). For the software to recognize genotypes, two non-template controls were included in each of 384-well plates. All samples were arrayed together in four 384-well plates, and the fifth plate contained eight duplicate samples from each of four plates to ensure the quality of genotyping (the concordance was >99% for all SNPs).

Immunohistochemistry

Tissue microarray (TMA) recipient blocks were constructed containing paraffin-embedded cancer tissues from 89 archival patient specimens by a tissue Array (Minicore; Alphelys Impasse Paul Langevin, Plaisir, France). The TMA blocks were cut into 4 μm thick continuous sections and mounted on poly-L-lysine-coated glass slides. Slides were deparaffinized in xylene, rehydrated in graded alcohols and washed in tap water. Prior to a primary antibody staining, the slide was pretreated with ethylenediaminetetraacetic acid buffer in a pressure cooker for antigen retrieval. Endogenous peroxidase activity was quenched by 3% H_2O_2 blocking reagent for 10 min. The slide was incubated with *DNMT3a* antibody (H-295, sc-20703, Santa Cruz, CA, USA; dilution 1:200) at 4 °C overnight, and then immunostained with the avidin-biotin peroxidase complex (DAKO, CA, USA). Finally, the slide was stained with diaminobenzidine according to the manufacturer's protocol (DAKO, CA, USA). The slide was rinsed three times with phosphate buffered saline after each step of staining. The stained

Table 1. Characteristics of the Subjects

	GC, n (%)	GA, n (%)	Controls, n (%)	*P* value
Number	447	111	961	
Sex				
Male	322(72.0)	66(59.5)	564(58.7)	<0.001
Female	125(28.0)	45(40.5)	397(41.3)	
Age				
≤45	36(8.1)	20(18.0)	285(29.7)	<0.001
46-65	239(53.5)	81(73.0)	607(63.2)	
> 65	172(38.5)	10(9.0)	69(7.2)	
H.pylori infection				
Negative	138(30.9)	27(24.3)	483(50.3)	<0.001
Positive	309(69.1)	84(75.7)	478(49.7)	
DNMT3a				
rs1550117				
GG	289(64.7)	71(64.0)	640(66.6)	0.586
AG	142(31.8)	33(29.7)	288(30.0)	
AA	16(3.6)	7(6.3)	33(3.4)	
rs13420827				
CC	295(66.0)	79(71.2)	618(64.3)	0.651
CG	133(29.8)	29(26.1)	304(31.6)	
GG	19(4.3)	3(2.7)	39(4.1)	
TNM staging				
I	64(14.3)			
II	181(40.5)			
III	144(32.2)			
IV	58(13.0)			
Differentiation				
Well	3(0.7)			
Moderate	187(41.8)			
Poor	257(57.5)			
Lauren classfication				
Diffuse	130(29.1)			
Intestinal	305(68.2)			
Mixed	12(2.7)			

slides were evaluated by two independent pathologists who were blinded for clinical data. The widely accepted HSCORE system that considered both the staining intensity and the percentage of cells stained at the specific range of intensity was adapted. The HSCORE was calculated following the equation: HSCORE=∑Pi(i) i=0, 1, 2, 3, Pi= 0~100%, where i is the intensity of the stained tumor cells(no staining=0, weak staining=1, moderate staining=2 and strong staining=3) and Pi is the percentage of stained tumor cells for each intensity varying from 0 to 100%. The HSCORE ranged from 0 to 300.

Statistics analysis

Deviations of genotype frequencies in controls from those expected under the Hardy-Weinberg equilibrium were assessed by a goodness-of-fit x^2-test. Linkage disequilibrium (LD) between pairs of biallelic loci was determined using two measures, D´ and r^2. Either Chi-square test or Fisher's exact test was performed by comparing distributions of genotype frequencies between patients and controls. Risks associated with rare genotypes were estimated as odds ratios (ORs). Corresponding 95% confidence intervals (CIs) were adjusted by age (scale variable), sex (nominal variable) and *H.pylori* antibody (nominal variable) by unconditional logistic regression. The association of *DNMT3a* expression with polymorphisms was analyzed by the Kruskal-Wallis H test and Fisher's exact test. All statistical tests were two-tailed and *P* values less than 0.05 were considered to be statistically significant. All analyses were performed using statistical software for windows SAS version 9.2 (SAS Institute, Cray, NC, USA). The power of the statistical tests was calculated using the QUANTO Version 1.2.3 software program (Jim Gauderman and John Morrison, University of Southern California, CA, USA).

Table 2. ORs and 95% CIs of *DNMT3a* Polymorphisms for *H.pylori* Infection in Control Group

Genotype	*H.pylori*(+) (%) (n=478)	*H.pylori*(-) (%) (n=483)	OR(95%CI)*	*P* value
rs1550117				
GG	65.5	67.7	Reference	
AG	29.9	30.0	1.03(0.78-1.36)	0.85
AA	4.6	2.3	2.02(0.96-4.24)	0.06
GG/AG	95.4	97.7	Reference	
AA	4.6	2.3	2.08(1.03-4.32)	0.04
G allele	80.4	82.7	Reference	
A allele	19.6	17.3	1.16(0.92-1.46)	0.20
rs13420827				
CC	65.5	63.1	Reference	
CG	29.9	33.3	1.24(0.64-2.38)	0.53
GG	4.6	3.5	0.87(0.66-1.14)	0.31
C allele	80.4	79.8	Reference	
G allele	19.6	20.2	0.96 (0.76-1.20)	0.73

*OR for each genotype and allele were calculated by age and sex adjusted

Results

Allele frequencies of the SNPs

A total of 447 patients with GC (322 males and 125 females, aged between 35 to 80 years old), 111 subjects with GA and 961 healthy subjects who passed health checks were recruited in this study. The characteristics of subjects are summarized in Table 1. The mean age was older in gastric cancer patients compared to the control group (61.6 vs. 50.6 years; $P < 0.001$). There were more females in the control group ($P < 0.001$). Prevalence rates of *H.pylori* seropositivity were significantly higher in the GC and GA groups compared to the control group (69.1%, 75.7% vs. 49.7%, $P<0.001$). Genotype distributions for rs1550117 and rs13420827 in the control group were consistent with the Hardy-Weinberg equilibrium ($P = 0.66$ and $P = 0.51$, respectively). No statistically significant differences were found between groups for the SNPs ($P =0.59$ and $P =0.65$, respectively). The linkage disequilibrium structure of these two polymorphic loci suggested that rs1550117 had very lower D' and r^2 with rs13420827 (0.07 and 0.14, respectively), therefore it was not necessary to assess the *DNMT3a* haplotypes.

Associations of SNPs with H.pylori seropositivity, GA and GC

Rates of *H.pylori* seropositivity were statistically significantly increased in subjects bearing with rs1550117 AA genotype compared to GG/AG genotypes (66.7% vs. 49.1%, *P* value=0.048). After adjusted by age and sex, the ORs for *H.pylori* seropositivity of AA genotype were 2.08 (95%CI: 1.02-4.32) relative to the GG/AG genotypes,

Table 3. ORs and 95% CIs of *DNMT3a* Polymorphisms for Gastric Atrophy and Gastric Cancer

Genotype	GA(%) n=111	Control(%) n=961	OR(95%CI)	*P* value	GC(%) n=447	Control(%) n=961	OR(95%CI)	*P* value
rs1550117								
GG	64.0	66.6	Reference		64.7	66.6	Reference	
AG	29.7	30.0	1.05(0.68-1.64)	0.82	31.8	30.0	1.10(0.83-1.47)	0.51
AA	6.3	3.4	1.71(0.72-4.11)	0.23	3.6	3.4	0.93(0.45-1.90)	0.84
G allele	88.8	91.2	Reference		80.5	81.6	Reference	
A allele	21.2	18.4	1.19(0.83-1.67)	0.33	19.5	18.4	1.06(0.83-1.32)	0.51
rs13420827								
CC	71.2	64.3	Reference		66.0	64.3	Reference	
CG	26.1	31.6	0.60(0.18-2.03)	0.42	29.8	31.6	1.07(0.55-2.10)	0.84
GG	2.7	4.1	0.81(0.51-1.27)	0.36	4.3	4.1	1.06(0.79-1.41)	0.71
C allele	84.2	80.1	Reference		80.9	80.1	Reference	
G allele	15.8	19.9	0.79(0.53-1.14)	0.13	19.1	19.9	1.05(0.83-1.33)	0.64

OR for each genotype and allele were calculated by age, sex and *H.pylori* seropostivity adjusted

Table 4. Influence of *DNMT3a* Polymorphisms on Expression of *DNMT3a* in Gastric Cancer

Genotype	Expression level, n(%)				Median HSCORE (quartile)	*P* value
	Negative	Low	Moderate	High		
rs1550117						
GG	6(10.9)	4(7.3)	10(18.2)	35(63.6)	180(60-240)*	0.09
AG	5(16.1)	2(6.5)	7(22.6)	17(54.8)	140(50-210)	
AA	1(33.3)	0(0)	1(33.3)	1(33.3)	30 (0-140)	
rs13420827						
CC	6(11.8)	3(5.9)	10(19.6)	32(62.7)	150(60-210)	0.16
CG	6(18.8)	2(6.3)	7(21.9)	17(53.1)	170(38-210)	
GG	0(0)	1(16.7)	1(16.7)	4(66.7)	165(91-240)	

*Median (min-max)

showing a recessive effect of the rare allele. No significant association was found between rs13420827 polymorphism and the *H.pylori* seropositivity (Table 2).

The age-, sex- and *H.pylori* antibody-adjusted ORs for GA were calculated, however, no association between two polymorphisms and the risk of GA was identified using unconditional logistic analysis (Table 3). Relationships of the SNPs with GC were also examined, but no significant correlation was found either (Table 3). The results of subgroup analysis did not suggest that sex, age and *H.pylori* status were effect modifier of the association between GA or GC and *DNMT3a* polymorphisms (data not shown). No relation was found between the SNPs and Lauren's classification, tumor differentiation or TNM staging (data not shown).

Association between SNPs and expression of DNMT3a

The results of immunohistochemstry staining are shown in Table 4. The strong nuclear expression of *DNMT3a* was shown in most of slides and a faint cytoplasm staining was only seen in a few slides. The *DNMT3a* expression is graded into four categories, high expression (HSCORE>200), moderate expression (100<HSCORE≤200), low expression (HSCORE≤100) and negative (HSCORE=0). The grades of high, moderate, low and negative expressions were detected in 53 (59.5%), 18 (20.2%), 6 (6.7%) and 12 (13.5%) gastric cancer tissues respectively. No differences of *DNMT3a* expression were seen between genotypes of rs1550117 and rs13420827 (*P* values were 0.09 and 0.16 respectively). To gain an insight of aberrant *DNMT3a* expression induced by *H.pylori* infection, we analyzed the expression level of *DNMT3a* in patients with and without *H.pylori* infection (62 vs. 27patients). No increase in the *DNMT3a* expression was observed in the patients with *H.pylori* infection (*P* value = 0.44).

Discussion

H.pylori infection has been generally accepted as an important factor in gastric carcinogenisis. Previous studies have shown that *H.pylori* infection could induce aberrant DNA methylation at the CpG islands, resulting in inactivations of tumor suppressor genes in gastric mucosa, and creating predisposed fields for cancerization (Maekita et al., 2006; Nakajima et al., 2006; Tsang et al, 2011). In contrast, a study reported that *H.pylori* infection did not induce either mRNA or protein expression of DNMT1, *DNMT3a* and DNMT3B in gastric mucosa directly (Nakajima et al., 2009). It has been hypothesized that SNPs of DNMTs may affect the methyltransferases levels of expression, involving in tumorgenesis and tumor progression.

Several SNPs of the *DNMT3a* gene have been identified, which may affect catalytic activity of the *DNMT3a* enzyme. For example, the rare A allele of the rs1550117 polymorphism in promoter was found to significantly increase DNTM3a transcriptional activity and was associated with an increased risk for GC and colorectal cancer (Fan et al., 2010; Zhao et al., 2012). Moreover, subjects carrying the CG heterozygosity of rs13420827 in 3'UTR was reported with a decreased risk for GC (Yang et al., 2012). However, we did not find any associations between *DNMT3a* polymorphisms and risk for GA or GC, but the subjects bearing rs1550117 AA genotype had significantly increased a risk of *H.pylori* infection in our study.

So far the relation between the variants of *DNMT3a* and *H.pylori* infection has not been investigated. A previous work has shown that MUC1, a membrane bound mucin expressed on the surface of gastric epithelial cells, provides a protective barrier against *H.pylori* infection. MUC1 gene expression is regulated by DNA methylation, for instance, MUC1 mRNA expression in MUC1-negative cells was restored by the treatment with

the DNA methylation inhibitor (Yamada et al., 2008). The luciferase assay demonstrated that the promoter activity of rs1550117 A allele of *DNMT3a* was significantly higher (more than two fold) than the G allele in Fan's study (Fan et al., 2010). Therefore, we can propose that *DNMT3a* rs1550117 G→A transition might increase the expression of *DNMT3a* and induce transcriptional repression of MUC1, resulting in the attenuation of MUC1 protective function. Further studies of *DNMT3a* sequence variants and their biologic functions may shed light on biological importance of the *DNMT3a* polymorphism in *H.pylori* infection.

Fan et al. (2010) recently found that rs36012910 polymorphism, which located at -2720 bp from the transcription start site in promoter associated with genetic susceptibility to GC (Fan et al., 2010; Wu et al., 2012). However they did not directly confirm that the rs36012910 polymorphism affects the activity of the *DNMT3a* promoter. The relationship between rs36012910 A>G polymorphism and risk of GC, may be due to the linkage disequilibrium with functional polymorphism rs1550117. In Yang's and our studies, no any relationships between rs1550117 with susceptibilities to GC were observed (Yang et al., 2012). Other studies also failed to find significant associations between tag SNPs of *DNMT3a* and breast cancer risk, or ovarian carcinoma (Mavaddat et al., 2009; Kelemen et al., 2010). Although Yang et al examined the associations of 5 SNPs from *DNMT3a* (rs1550117, rs11887120, rs13420827, rs13428812, rs6733301) with GC in the Southern Chinese population, there was a significant protective effect between GC heterozygosity in rs13420827 and GC in the overdominant model, with OR = 0.66 (0.45–0.97) (Yang et al., 2012). We were unable to find the similar observed association between rs13420827 with risk of GC. The inconsistent results from these studies may be caused by different selection of study subjects and environmental backgrounds, such as age, smoke, and *H.pylori* infection group. In our study, GC cases were non-cardiac GC patients, and in Yang's studies, GC cases include non-cardiac GC and cardiac GC patients. It has been long thought that differences of etiology between non-cardiac GC and cardiac GC. In addition, we have selected cancer-free healthy examinees as healthy controls, and Yang et al selected cancer-free outpatients and inpatients as hospital controls, thus, the discordance of results may result from sampling. Furthermore, in our study, *H.pylori* infection as an important influencing factor for GC was adjusted and stratified in the analysis, which may have led to preferably explore the association between the *DNMT3a* polymorphisms and risk of GC. The statistical power is sufficient (power > 0.99) to examine the OR > 2.0 association between SNPs and risk of GC in the present study.

In addition, the over-expression of *DNMT3a* mRNA and protein were detected in various cancers including GC, these results revealed that increased *DNMT3a* expressiom may play a role in tumorgenesis of GC (Oh et al., 2007; Ding et al., 2008; Rahman et al., 2010; He et al., 2011; Yang et al., 2011). However, no differences of *DNMT3a* expression was detected between different genotype carriers in GC patients in our study. *DNMT3a* mutations and allelic losses have been reported in many solid cancers (Kim et al., 2013), which decreased the emzymatic activity of encoded protein, suggesting that the abnormal expression or accumulation of *DNMT3a* in cancer tissues may be due to defects in the degradation of mutant products and no relation with the polymorphisms.

In summary, we did not find significant associations for *DNMT3a* rs1550117 and rs13420827 polymorphisms with GA or GC, but our finding provides the first evidence of association between rs1550117 and increased risks for *H.pylori* infection in the Chinese population. Further investigations are required to fully understand the biological importance of these polymorphisms.

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