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Genetic Variation in *A4GNT* in Relation to *Helicobacter pylori* Serology and Gastric Cancer Risk

Zongli Zheng[1], **Yanbin Jia**[1,2], **Lifang Hou**[3,4], **Christina Persson**[1], **Meredith Yeager**[5], **Jolanta Lissowska**[6], **Stephen J. Chanock**[5], **Martin Blaser**[7], **Wong-Ho Chow**[3], and **Weimin Ye**[1,*]

[1] Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden [2] Department of Basic Medicine, Baotou Medical College, Baotou, China [3] Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD [5] Core Genotyping Facility, Advanced Technology Center, National Cancer Institute, Gaithersburg, MD [6] Department of Cancer Epidemiology and Prevention, The M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland [7] Department of Medicine, New York University School of Medicine, New York, NY

Abstract

OBJECTIVES—*Helicobacter pylori* (*H. pylori*), a known risk factor of gastric cancer, rarely colonize the deeper portion of normal gastric glands, where the mucus is rich in alpha-1,4-linked N-acetylglucosamine (A4GN) capped O-glycans, that strongly inhibit *H. pylori* growth *in vitro*.

METHODS—We investigated the association between genetic variation in the O-glycan transferase encoding gene (*A4GNT*) and *H. pylori* infection and gastric cancer risk using a Polish population-based case-control study (273 gastric cancer patients and 377 controls).

RESULTS—A haplotype at the rs2622694-rs397266 locus was associated with *H. pylori* infection, with the A-A haplotype associated with higher risk compared with the most frequent G-G haplotype (odds ratio 2.30; 95% confidence interval 1.35 – 3.92). The association remained significant after correction for multiple tests (global *P* value: nominal 0.002, empirical 0.045). Neither this haplotype nor the tagSNPs were associated with overall gastric cancer risk.

CONCLUSION—*A4GNT* genetic variation may be relevant to *H. pylori* infection, but not to gastric cancer risk.

[*] **Correspondence to:** Weimin Ye Department of Medical Epidemiology and Biostatistics Karolinska Institutet Box 281 SE-17177 Stockholm Sweden weimin.ye@ki.se.
[4]Current address: Northwestern University Feinberg School of Medicine, Chicago, IL

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CONFLICT OF INTEREST
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Specific author contributions:
Conception and design: Weimin Ye, Zongli Zheng, Wong-Ho Chow, Meredith Yeager;
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Critical revision of manuscript: Yanbin Jia, Lifang Hou, Christina Persson, Jolanta Lissowska, Martin Blaser;
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INTRODUCTION

Gastric cancer remains the second leading cause of cancer-related death worldwide (1). *Helicobacter pylori* (*H. pylori*) infection is the most important acquired risk factor for gastric cancer (2). The past decade has seen many studies on genetic susceptibility to gastric cancer, particularly for genes in the inflammatory and detoxifying pathways (3-5). Few studies of genetic susceptibility have focused on host defenses against *H. pylori* within the gastric mucus (4,5).

The gel layer covering the gastric mucosa is the major reservoir of *H. pylori*. An uneven distribution of *H. pylori* among mucins of varied physiochemical properties, as observed by electron microscopy (6), provides clues to understand how the host environment may influence *H. pylori* survival. *H. pylori* rarely colonize the deeper portions of the normal gastric mucosa (6,7), where the mucins are rich in O-glycans capped with terminal alpha-1,4-linked N-acetylglucosamine (A4GN) (8,9). Glycans possessing the A4GN residue, but not those without, suppressed *H. pylori* growth in vitro (10,11). The transfer of A4GN to beta-Gal residues with alpha1,4-linkage, forming GlcNAc alpha-1, 4-Gal beta-R structures, is mediated by a transferase encoded by the gene *A4GNT* (8). We hypothesized that genetic variation in *A4GNT*, which could lead to varied expression or function of this transferase, may be related to the ability of *H. pylori* to establish colonization, and consequent gastric cancer risk.

METHODS

Study design

A population-based case-control study on gastric cancer was conducted in Warsaw, Poland between 1994 and 1996, as described (12,13). Cases (n=464) were patients newly diagnosed with histologically confirmed gastric cancer. Controls (n=480) were randomly selected from the computerized population registry and frequency matched to the cases by sex and age (±5 years). Blood samples were obtained from 305 (65.7%) cases and 427 (90.0%) controls. Genomic DNA was available from 273 cases and 377 controls. Informed consent was obtained from each participant. The study was approved by Institutional Review Boards at the US National Cancer Institute, the Cancer Center and Institute of Oncology of Health in Warsaw, Poland, and Regional Ethics Committee of Karolinska Institutet in Sweden.

SNP tagging and genotyping

A total of 59 single nucleotide polymorphisms (SNPs) spanning the *A4GNT* gene and flanking region (100kb) with minor allele frequency ≥ 5% were identified from the HapMap2 – CEU population database (http://www.hapmap.org/). SNPs were tagged using the Carlson approach such that a resulting tagSNP had $r^2 \geq 0.8$ with all other SNPs grouped in the same bin (14). A total of 59 SNPs were grouped into 9 bins, yielding 9 tagSNPs for assay by the SNPlex Genotyping System (Applied Biosystems 3730 DNA Sequencer) at Core Genotyping Facility of National Cancer Institute, MD. Two (rs2622723, rs329379) failed during the genotyping process, since they were singleton-tags, lacking information about other SNPs. The remaining seven tagSNPs (rs996432, rs405265, rs11928535, rs329386, rs2246945, rs2622694, rs397266), which carry information on 57 SNPs across the 100kb region, were available for final analysis. Details about assays, primers, probes, and procedures are available on the National Cancer Institute's SNP500 website (http://snp500cancer.nci.nih.gov).

Assay for antibodies against *H. pylori* and CagA

Serum levels of IgG antibodies against *H. pylori* whole cell antigen and antibodies against CagA were measured using ELISA, as described (15).

Statistical analysis

Hardy–Weinberg equilibrium was tested using Pearson's χ^2-test in cancer-free controls. In single locus analysis, we compared different genotypes, with the most common genotypes among *H. pylori*-negative persons or controls served as references, and performed the Armitage trend tests. Because circulating CagA antibody is able to reflect infection even with negative values in the whole cell assay (16,17), we defined *H. pylori* infection by positivity in anti-*H. pylori* and/or CagA assays. Odds ratios (ORs) with 95% confidence intervals (95% CIs) derived from unconditional logistic models were used to assess relative risks. All estimates were adjusted for sex and age. Firth's penalized maximum likelihood estimation was used in case of data separation (18). A priori, we also performed stratified analyses by anatomic location (cardia / non-cardia) and histological classification (intestinal / diffuse) of the tumour. To account for increased type I errors of multiple testing, statistical significance was assessed by empirical *P* values derived from Westfall and Young permutation (n=10,000) with minimum *P* and step-down method(19), where case-control status of all subjects were randomly permuted, then the set of seven SNPs analyses were performed for each permutation dataset, and the minimal *P* value of the seven analyses were recorded. The distribution of the 10,000 minimal *P* values obtained from 10,000 permutation datasets was used to derive the empirical significance of the observed test statistic (empirical *P* value equals to the percentile of the observed *P* in the distribution of the 10,000 minimal *P* values).

Haplotypes were inferred with comparison groups (infected vs. uninfected, or cancer cases vs. controls) jointly by an Expectation-Maximization algorithm, and analyzed by a “sliding-window” approach with varied window sizes ranging from 2 to 4 tagSNPs (20). Rare haplotypes, i.e. < 1% among the controls, were combined as one group. The probabilities, inferred from the EM algorithm, of having certain haplotypes for each individual were used as weights in a logit binomial model with sandwich covariance (21). The most common haplotype among uninfected or controls was used as a reference for each sliding window. Global *P* values were used to assess the difference in haplotype profiles between comparison groups. Permutations (n=10,000) were conducted to adjust global *P* values of multiple tests for all sliding windows. All analyses were conducted using the SAS 9.2 (SAS Institute, Cary, NC) package. (A SAS macro for sliding-window haplotype construction and permutation is available from the author)

RESULTS

All seven tested SNPs were in Hardy-Weinberg equilibrium (Table 1). Age and gender distributions were comparable between the case (mean age 63.4 ± 10.5, male 67.8%) and control (mean age 63.0 ± 10.3, male 65.0%) groups, as these were frequency matching factors. Compared with the controls, gastric cancer cases were more likely to have lower education, smoke more, and have a familial history of gastric cancer (data not shown). The majority of cases had the intestinal histological type (69.1%) and originated from the non-cardia region of the stomach (72.2%).

A4GNT and *H. pylori* infection, single-locus analysis

SNP rs2622694 heterozygous genotype AG was associated with a 49% reduced risk of *H. pylori* infection compared with the most common genotype AA (OR 0.51, 95% CI 0.26-1.01, Table 2). A similar magnitude of reduced risk was observed for the homozygous

variant genotype GG (OR 0.52, 95% CI 0.21-1.31). For SNP rs397266, the heterozygous genotype AG conferred a 68% higher risk of *H. pylori* infection than the most common genotype GG. All carriers (n=29) of the homozygous variant genotype AA had *H. pylori* infection. Armitage trend test revealed a *P* value of 0.003, which remained significant after correction for multiple tests (0.036).

A4GNT and *H. pylori* infection, haplotype analysis

Among the fifteen sliding windows, three (rs2622694-rs397266, rs2246945-rs2622694-rs397266 and rs329386- rs2246945-rs2622694-rs397266) had haplotype profiles that differed between the infected and uninfected groups (Table 3). These three windows all pointed to a significant effect of haplotype at loci rs2622694-rs397266. Compared with the most common (G-G) haplotype, haplotype A-A was associated with a significantly higher risk of *H. pylori* infection (OR 2.30, 95% CI 1.35-3.92, global *P* 0.0019). This association remained significant after correction for multiple tests (empirical global *P* value 0.045).

A4GNT and gastric cancer, single-locus analysis

Overall, none of the tested SNPs was associated with gastric cancer risk (Table 4). When limiting to the intestinal type of gastric cancer, the SNP rs2622694 G allele was associated with a reduced risk compared with the A allele (nominal trend test *P* value 0.029, data not shown); GG carriers had only half the risk compared with AA carriers (OR 0.53; 95% CI 0.28-1.01). However, the association was non-significant after correction for multiple tests (empirical trend test *P* value 0.24). For the diffuse type gastric cancer, despite the small sample size (n = 46), SNP rs996432 T allele was associated with a higher risk compared with C allele (TT vs. CC: OR 2.51, 95% CI 1.01-6.20), but became non-significant after correction for multiple tests (data not shown). Stratified analyses by tumour location did not reveal materially different results between cardia and distal gastric cancer (data not shown).

A4GNT and gastric cancer, haplotype analysis

Similarly as for single locus analysis, haplotypes at the seven loci were not associated with gastric cancer risk overall (data not shown). In stratified analyses, the haplotypes constructed from rs329386-rs2246945-rs2622694 loci were borderline associated with the intestinal type of gastric cancer (global *P* value 0.057). Compared with the most frequent haplotype C-A-A (59.2% among controls), haplotype C-C-G (31.4% among controls) was associated with a non-significant (25%) reduced risk for intestinal type gastric cancer (OR 0.75, 95% CI 0.56 - 1.01), but haplotype G-C-G (6.4% among controls) was associated with a 50% risk reduction (OR 0.50, 95% CI 0.27-0.94). However, the global *P* value was non-significant in the permutation test (empirical *P* value 0.24), though. For the diffuse type of gastric cancer, none of the haplotypes was associated with risk (data not shown). Stratifying the analysis by tumour location did not reveal material differences.

DISCUSSION

Host genetic variation was estimated to contribute 57% of variation in acquisition of *H. pylori* infection (22). No prior epidemiological study had examined the role of variation in *A4GNT* in relation to risk of *H. pylori* infection or gastric cancer. Evidence from in vitro studies demonstrates that O-glycans capped with the residue A4GN alpha-1, 4Gal beta is capable of exerting an inhibitory effect on *H. pylori* growth by inhibiting the biosynthesis of cholesteryl-alpha-D-glucopyranoside, a major *H. pylori* cell wall component (10,11,23). The transfer of A4GN to betaGal with alpha1,4-linkage, forming A4GN alpha-1, 4-Gal beta-R structures, is mediated by a transferase encoded by *A4GNT* [9]. In contrast to an expected beneficial effect of the A4GNT gene product, two small clinical series studies showed up-regulated expression of *A4GNT* mRNA in patients with gastric cancer (24). One explanation

for this discrepancy may be that there exist functionally impotent subclasses of the A4GNT-encoded enzyme. This is supported by the observation that the expression of the A4GNT enzyme, but not of the A4GNalpha1-4Galbeta-R (a mucous gland specific mucin specific glycan that suppresses *H. pylori* growth), was up-regulated amongst patients with *H. pylori* gastritis and decreased to normal level after *H. pylori* eradication (7). The up-regulation of A4GNT enzyme accompanied by unchanged amount of A4GNalpha1-4Galbeta-R suggests that there exist subclasses of A4GNT with little or no transferase activity. Hosts with up-regulated expression of non-functional A4GNT subclasses would be less capable of preventing *H. pylori* from establishing colonization and, consequently, could have more severe clinical outcomes, such as gastric cancer (24).

Examining into the sequence of A4GNT (8) that had been used in the previous *in vitro* studies (10,11), in which the A4GNT, producing a glycan suppressing *H. pylori* growth, had an alanine at position 218 (corresponding to a C allele at rs2246945), consistent with our finding that the C allele rs2246945 was associated with reduced risk for *H. pylori* infection. Since the effect of A4GNT only has been demonstrated in one sequence that came from the same research group, functional studies on subclasses of A4GNT encoded by other sequences are warranted.

In our study, *A4GNT* variation was associated with *H. pylori* seropositivity but was not associated with overall gastric cancer risk. The proportion of the risk haplotype A-A at loci rs2622694-rs397266 was 17% among the uninfected group in this study. Such a relatively low prevalence requires a larger sample-size study to examine for moderate or modest effects of *A4GNT* on gastric cancer risk.

In summary, this preliminary study, suggests that the A-A haplotype at rs2622694-rs397266 may be related with an increased risk for *H. pylori* infection, but no strong effect on gastric cancer risk was observed.

STUDY HIGHLIGHTS

What Is Current Knowledge

- A4GNT has an anti-*Helicobacter pylori* effect *in vitro*.
- *A4GNT* gene is polymorphic in population.
- Whether the polymorphism of *A4GNT* is related to *Helicobacter pylori* infection in the population and how this further relates with gastric cancer development is unknown.

What Is New Here

- These data indicate that some variants of *A4GNT* are related to *Helicobacter pylori* infection in the population.
- However, this relation does not affect the development of gastric cancer.

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Table 1

Contig position, function, allele frequency and Hardy–Weinberg equilibrium tests of the *A4GNT*[*] tagSNPs among controls

dbSNP ID	Gene symbol [*]	Contig position [†]	Function annotation (protein systematic)	Minor allele, (frequency, %)[‡]	HWE test *P* value [‡]
rs996432	DZIP1L	44303606	Intro_5 −1111T>C	T (35.6)	0.62
rs405265	DZIP1L	44309849	Intro_3 −878T>G	T (15.7)	0.62
rs11928535	DZIP1L	44313245	Intro_2 −1410C>T	T (20.2)	0.17
rs329386	DZIP1L	44329244	Intro_1 +72C>G	G (8.0)	0.78
rs2246945	A4GNT	44338622	Exon_3 +245C>A (Ala218Asp)	C (37.9)	0.83
rs2622694	A4GNT	44344015	Intro_2 +822G>A	G (38.2)	0.70
rs397266	A4GNT	44355253	20623G>A	A (28.9)	0.55

[*] Based on NCBI Entrez database, alpha-1,4-N-acetylglucosaminyltransferase (*A4GNT*), DAZ interacting protein 1-like (*DZIP1L*)

[†] Based on Contig NT_005612

[‡] Among controls

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Table 2

Association between *A4GNT* tagSNPs and *H. pylori* infection (defined by positivity in anti-*H. pylori* and/or CagA assays) among 376 controls from a Polish gastric cancer case-control study

SNP	Genotype	*H. pylori* negative, n (%)	*H. pylori* positive, n (%)	OR*	Trend *P* nominal	Trend *P* empirical†
SNP1: rs996432	CC	17 (32.1)	134 (42.1)	1.0 (reference)	0.16	0.67
	CT	28 (52.8)	147 (46.2)	0.63 (0.33-1.22)		
	TT	8 (15.1)	37 (11.6)	0.58 (0.23-1.47)		
SNP2: rs405265	GG	35 (63.6)	230 (71.9)	1.0 (reference)	0.20	0.72
	GT	18 (32.7)	84 (26.3)	0.70 (0.37-1.31)		
	TT	2 (3.6)	6 (1.9)	0.53 (0.10-2.87)		
SNP3: rs11928535	CC	34 (60.7)	201 (62.8)	1.0 (reference)	0.45	0.91
	CT	19 (33.9)	111 (34.7)	0.94 (0.51-1.73)		
	TT	3 (5.4)	8 (2.5)	0.44 (0.11-1.80)		
SNP4: rs329386	CC	49 (87.5)	267 (84.2)	1.0 (reference)	0.42	0.90
	CG	7 (12.5)	48 (15.1)	1.25 (0.54-2.90)		
	GG	0 (0.0)	2 (0.6)	0.97 (0.02-41.47)		
SNP5: rs2246945	AA	16 (28.6)	130 (40.8)	1.0 (reference)	0.11	0.52
	AC	30 (53.6)	144 (45.1)	0.55 (0.28-1.07)		
	CC	10 (17.9)	45 (14.1)	0.55 (0.23-1.33)		
SNP6: rs2622694	AA	14 (26.4)	122 (39.7)	1.0 (reference)	0.09	0.49
	AG	30 (56.6)	143 (46.6)	0.51 (0.26-1.01)		
	GG	9 (17.0)	42 (13.7)	0.52 (0.21-1.31)		
SNP7: rs397266	GG	37 (66.1)	150 (47.2)	1.0 (reference)	0.003	0.04
	AG	19 (33.9)	139 (43.7)	1.68 (0.92-3.07)		
	AA	0 (0.0)	29 (9.1)	16.7 (0.98-286.2)		

*Adjusted for sex and age.

†For each permutation, all genotype comparisons and trend tests were taken into account (i.e. 21 tests in each permutation). The number of permutations was 10,000.

Table 3

Association between *A4GNT* haplotypes and *H. pylori* infection (defined by positivity in anti-*H. pylori* and/or CagA assays) among 376 controls from a Polish gastric cancer case-control study

Haplotype window*	Haplotype	*H. pylori* negative, frequency (total 56)	*H. pylori* positive, frequency (total 320)	OR †	Global *P*, nominal	Global *P*, empirical‡
SNP6to7	G-G	0.45	0.37	1.0 (reference)	0.002	0.045
	A-G	0.38	0.32	1.05 (0.68-1.62)		
	A-A	**0.17**	**0.310**	**2.30 (1.35-3.92)**		
SNP5to7	C-G-G	0.43	0.36	1.0 (reference)	0.014	0.22
	A-A-G	0.36	0.32	1.07 (0.68-1.67)		
	A-A-A	**0.17**	**0.31**	**2.23 (1.31-3.79)**		
	A-G-G	0.02	0.01	0.66 (0.14-3.16)		
	C-A-G	0.02	0.003	0.21 (0.04-1.16)		
SNP4to7	C-C-G-G	0.38	0.31	1.0 (reference)	0.031	0.36
	C-A-A-G	0.36	0.32	1.12 (0.70-1.79)		
	C-A-A-A	**0.16**	**0.29**	**2.29 (1.33-3.94)**		
	G-C-G-G	0.06	0.07	1.53 (0.63-3.69)		
	C-A-G-G	0.02	0.01	0.64 (0.13-3.07)		
	C-C-A-G	0.02	0.003	0.21 (0.04-1.20)		

*SNP 1 to 7: rs996432, rs405265, rs11928535, rs329386, rs2246945, rs2622694 and rs397266. In total, there are 15 windows with sizes ranging from 2 to 4 tagSNPs. Only those haplotype windows with global $P < 0.05$ were listed.

†Adjusted for sex and age.

‡Based on global P values of the total 15 haplotype windows. The number of permutations was 10,000.

Table 4

Association between *A4GNT* tagSNPs and gastric cancer risk in a Polish case-control study

SNP	Genotype	Control (n = 376)	Case (n = 273)	OR*	Trend *P*, nominal
rs996432	CC	152 (40.9)	118 (43.7)	1.0 (reference)	0.59
	CT	175 (47.0)	120 (44.4)	0.88 (0.63-1.23)	
	TT	45 (12.1)	32 (11.9)	0.93 (0.56-1.56)	
rs405265	GG	266 (70.7)	196 (72.6)	1.0 (reference)	0.77
	GT	102 (27.1)	67 (24.8)	0.89 (0.62-1.28)	
	TT	8 (2.1)	7 (2.6)	1.24 (0.44-3.48)	
rs11928535	CC	236 (62.6)	174 (64.9)	1.0 (reference)	0.67
	CT	130 (34.5)	85 (31.7)	0.89 (0.63-1.24)	
	TT	11 (2.9)	9 (3.4)	1.10 (0.45-2.72)	
rs329386	CC	316 (84.5)	239 (88.5)	1.0 (reference)	0.11
	CG	56 (15.0)	31 (11.5)	0.74 (0.46-1.18)	
	GG	2 (0.5)	0 (0.0)	0.26 (0.01-10.56)	
rs2246945	AA	146 (38.8)	120 (44.1)	1.0 (reference)	0.13
	AC	175 (46.5)	121 (44.5)	0.84 (0.60-1.18)	
	CC	55 (14.6)	31 (11.4)	0.69 (0.42-1.15)	
rs2622694	AA	136 (37.7)	112 (44.6)	1.0 (reference)	0.07
	AG	174 (48.2)	112 (44.6)	0.78 (0.55-1.11)	
	GG	51 (14.1)	27 (10.8)	0.65 (0.38-1.11)	
rs397266	GG	187 (49.9)	129 (47.8)	1.0 (reference)	0.64
	AG	159 (42.4)	120 (44.4)	1.10 (0.79-1.53)	
	AA	29 (7.7)	21 (7.8)	1.06 (0.58-1.94)	

*Adjusted for sex and age.