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ORIGINAL ARTICLE

Genetic polymorphisms and tissue expression of interleukin-22 associated with risk and therapeutic response of gastric mucosa-associated lymphoid tissue lymphoma

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Chronic *Helicobacter pylori*-stimulated immune reactions determine the pathogenesis of gastric mucosa-associated lymphoid tissue (MALT) lymphoma. We aimed to explore the genetic predisposition to this lymphoma and its clinical implication. A total of 68 patients and 140 unrelated controls were genotyped for 84 single-nucleotide polymorphisms in genes encoding cytokines, chemokines and related receptors that play important roles in T cell-mediated gastrointestinal immunity. Five genotypes in *IL-22*, namely CC at rs1179246, CC at rs2227485, AA at rs4913428, AA at rs1026788 and TT at rs7314777, were associated with disease susceptibility. The former four genotypes resided in the same linkage disequilibrium block ($r^2 = 0.99$) that conferred an approximately threefold higher risk. *In vitro* experiments demonstrated that co-culturing peripheral mononuclear cells or $CD4^+$ T cells with *H. pylori* stimulated the secretion of interleukin-22 (IL-22), and that IL-22 induced the expression of antimicrobial proteins, RegIIIα and lipocalin-2, in gastric epithelial cells. Furthermore, patients with gastric tissue expressing IL-22 were more likely to respond to *H. pylori* eradication (14/22 vs 4/19, $P < 0.006$). We conclude that susceptibility of gastric MALT lymphoma is influenced by genetic polymorphisms in *IL-22*, the product of which is involved in mucosal immunity against *H. pylori* and associated with tumor response to *H. pylori* eradication.

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INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) lymphoma, the most common histological type of primary gastric lymphoma, is a marginal zone B-cell lymphoma with extranodal location in the stomach.[1,2] *Helicobacter pylori* infection is strongly associated with gastric MALT lymphoma.[3] Epidemiological studies have revealed that >90% of patients with gastric MALT lymphoma were infected with *H. pylori*, and laboratory work has shown that *H. pylori* could stimulate lymphoma cell growth through T cell-mediated mechanisms.[4,5] In addition, eradication of *H. pylori* may result in sustained remission and often cure of the disease.[6,7] All these lines of evidence indicate that *H. pylori* is essential in the pathogenesis of most gastric MALT lymphomas.

Long-standing antigenic stimulation is regarded as a major drive of *H. pylori*-related marginal zone B-cell lymphomagenesis in the stomach. Gastric mucosa chronically infected with *H. pylori* evokes the T helper type 1 (Th1) and Th17 immune responses accompanied with a variety of pro-inflammatory cytokines and chemokines,[8–12] leading to an immune-driven lymphoid proliferation and subsequently the formation of lymphoma.[13,14] However, only <0.01% of the infected patients eventually develop this rare malignant disease,[15] indicating that the clinical consequence of *H. pylori* infection results from a complex interplay among bacterial, environmental and host factors.[16,17] As chronic inflammation holds a central role in the pathogenesis of gastric MALT lymphoma, polymorphisms of genes regulating gastric mucosal immunity may influence the disease susceptibility. We have previously reported that polymorphisms in *TNF*-α, *GSTT1* and *CTLA4* genes might modify the risk of gastric MALT lymphoma.[18–20] In order to further elucidate the genetic predisposition to this rare disease, we focused in this study on the genetic variations of cytokines and chemokines that mediated gastrointestinal inflammation. With a systemic approach, we first investigated genes (*IL-12B*, *IL-17A*, *IL-17F*, *IL-17RA*, *IL-21*, *IL-21R*, *IL-22*, *IL-23A* and *IL-23R*) encoding cytokines/cytokine receptors that were important in T cell-mediated responses against *H. pylori* infection,[8–12,21] and those (*CCR6*, *CX3CR1*, *CXCR3*, *CXCR5*, *CCL20*, *CX3CL1*, *CXCL9*, *CXCL10* and *CXCL13*) encoding chemokines/chemokine receptors that regulated gastrointestinal mucosa immunity.[22–25] Among these genes, polymorphisms in *IL-22* were found to be significantly associated with gastric MALT lymphoma. We then explored the involvement of interleukin-22 (IL-22) in *H. pylori* infection. Finally, we examined whether tissue expression of IL-22 influenced therapeutic responses to *H. pylori* eradication.

MATERIALS AND METHODS

Patients and treatment course

A total of 68 ethnically Han Chinese patients were enrolled from several medical centers in Taiwan through a national project for

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revealing risk factors and natural history of gastric MALT lymphoma. All cases had been histopathologically confirmed. The control group comprised 140 unrelated individuals randomly selected from a health checkup program, who had no evidence of malignant diseases, autoimmune disorders or any other major illness. All participants voluntarily consented to participate in this study. The Department of Health, Executive Yuan, Taiwan, the Taiwan Cooperative Oncology Group for gastric MALT lymphoma and the institutional research board of National Taiwan University Hospital approved the study protocol (NTUH-9561703044).

H. pylori infection was screened serologically using a standard enzyme-linked immunosorbent assay and confirmed by histologic examination, biopsy urease test, urea breath test or bacterial culture. All patients positive for *H. pylori* received eradication therapy as the first-line treatment, and then underwent intensive endoscopic follow-up with biopsy to evaluate the response of lymphoma to successful *H. pylori* eradication. Tumors that resolved to Wotherspoon's score of $\leqslant 2$ after successful *H. pylori* eradication therapy were considered as complete histologic remission. Systemic chemotherapy was administered to those with grossly stable or progressive disease during the follow-up course.

Selection of candidate genes and single-nucleotide polymorphisms

We started from a case–control study with a two-step systemic approach to unravel genetic association with gastric MALT lymphoma. The first step was to screen 18 genes encoding cytokines and cytokine receptors that play important roles in T cell-mediated inflammatory responses in gastrointestinal mucosa (*IL-12B*, *IL-17A*, *IL-17F*, *IL-17RA*, *IL-21*, *IL-21R*, *IL-22*, *IL-23A* and *IL-23R*), or chemokines and chemokine receptors that regulate the recruitment of inflammatory cells to sites of infection in gastrointestinal mucosa (*CCR6*, *CX3CR1*, *CXCR3*, *CXCR5*, *CCL20*, *CX3CL1*, *CXCL9*, *CXCL10* and *CXCL13*). Information of single-nucleotide polymorphisms (SNPs) in each gene was collected from the HapMap database (http://hapmap.ncbi.nlm.nih.gov/). For genotyping, we selected a total of 84 SNPs that are located within the coding region, the 3′ and 5′ untranslated regions or 1 kb 3′ and 5′ flanking regions (Supplementary Appendix I and II). In the first step, we uncovered a significant association with polymorphisms in *IL-22*. To further delineate the association, we performed the second step of genotyping focusing on *IL-22*. The *IL-22* SNPs selected for additional genotyping analysis were those with a minor allele frequency of >0.05 in the Han Chinese population and were located within the exons, 3′ and 5′ flanking regions and intron 1. Totally, 35 *IL-22* SNPs were genotyped.

Genotyping methods

Genomic DNA was isolated from cryopreserved leukocytes by proteinase K digestion followed by phenol–chloroform extraction. Genotyping was performed by using the iPLEX assay and Sequenom matrix-associated laser desorption/ionization time-of-flight mass spectrometry according to the manufacturer's instructions (Sequenom, San Diego, CA). Briefly, we utilized the SpectroDESIGNER software (Sequenom) to design primers and probes for performing multiplex PCR. Single base primer extension was performed after deactivation of the remaining nucleotides by shrimp alkaline phosphatase. The products of primer extension were spotted on a 384-element silicon chip (SpectroCHIP, Sequenom) and assayed using the matrix-associated laser desorption/ionization time-of-flight SpectroREADER mass spectrometer (Sequenom).

Cell line and *H. pylori* culture

AGS cells (human gastric adenocarcinoma epithelial cell line) were maintained in RPMI-1640 medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2.

The *H. pylori* clinical isolate (NTUHC1) was obtained from the National Taiwan University Hospital and was grown on CDC anaerobe 5% sheep blood agar (BBL, Becton-Dickinson, Franklin Lakes, NJ, USA) at 37 °C under microaerophilic condition. After overnight culture, *H. pylori* cells were harvested, washed once and resuspended in phosphate-buffered saline. The bacteria were then diluted with cell culture medium for co-culture experiments.

Isolation of PBMCs and the co-culture of PBMCs with *H. pylori*

Elutriated leukocytes or buffy coats were collected from healthy donors at the Taipei Blood Center (Taipei, Taiwan). The leukocytes were diluted fivefold with phosphate-buffered saline, and peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA) gradient centrifugation. PBMCs were cultured in complete RPMI-1640 overnight and washed three times with complete RPMI-1640 (without penicillin/streptomycin), then resuspended in complete RPMI-1640 at a concentration of 5×10^5 cells/ml, and co-cultured with *H. pylori* at a multiplicity of infection of 30. After 48 h of co-culture, the culture supernatants were collected and assayed for IL-22 by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). The peripheral blood T cells were further isolated from PBMCs using an AutoMACS magnetic cell sorter (Miltenyl, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, PBMCs were resuspended in phosphate-buffered saline buffer containing 2% fetal bovine serum followed by the incubation with 2% mouse serum for 20 min at 4 °C to block Fcγ receptors. The cells were then incubated with anti-CD4 conjugated with fluorescein isothiocyanate (eBioscience, San Diego, CA, USA) or anti-CD8 antibody conjugated with phycoerythrin (eBioscience) for the purification of peripheral $CD4^+$ T cells and $CD8^+$ T cells, respectively. After 20-min of incubation at 4 °C, cells were washed twice with 2% fetal bovine serum/phosphate-buffered saline. The cells were then resuspended in MACS buffer at 10^8 cells/ml followed by incubation with anti-fluorescein isothiocyanate beads for the isolation of $CD4^+$ T cells or with anti-phycoerythrin beads for the isolation of $CD8^+$ T cells. After 15 min of incubation at 4 °C, cells were washed once and subjected to autoMACS separation for the isolation of $CD4^+$ and $CD8^+$ T cells. The isolated $CD4^+$ or $CD8^+$ T cells (5×10^5 cells/ml) were co-cultured with *H. pylori* in the presence of antigen-presenting cells, and the culture supernatants were collected for measuring IL-22 levels by enzyme-linked immunosorbent assay.

Stimulation of AGS with IL-22 and assay of RegIIIα and lipocalin-2 expression

AGS cells were seeded in 6-cm dishes and cultured in the absence or presence of IL-22 (20 ng/ml; R&D Systems) for 0, 6, 12, or 24 h before being harvested. Total RNAs were extracted using the Trizol reagent followed by first-strand complementary DNA synthesis (Invitrogen). The complementary DNA was mixed with Maxima SYBR Green qPCR Master Mix (Fermentas, Lithuania, Canada) and specific primer pairs. The mixture was subjected to SYBR real-time PCR using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The resultant PCR products were analyzed by ABI 7500 software (Applied Biosystems). The ΔΔCt method was used to quantify the levels of gene expression. The primers for genes were as follows: β-*actin*, forward: 5′-TGGACTTCGAGCAAG AGATG-3′, reverse: 5′-TTGCTGATCCACATCTGCTG-3′; *lipocalin-2*, forward: 5′-AAGACAA AGACCCGCAAAAGATG-3′, reverse: 5′-GTCCTGATCCAGTAGTCACACTTC-3′, *RegIIIα*, forward: 5′-CAGGGCACCGAGCCCAATGGAG-3′, reverse: 5′-TCTCTC CCATGCAAAGTAAT-3′.

Immunohistochemical staining of IL-22 in gastric mucosa

Immunohistochemical staining of IL-22 in gastric tissue was performed in all ($n=41$) patients who had available pretreatment histological specimens. Paraffin blocks containing the specimens were cut into 5 μm sections. The first section was routinely stained with hematoxylin and eosin for histology, and sequential sections were immunohistochemically stained for IL-22 with a standard avidin-biotin-peroxidase complex detection system. Endogenous peroxidase activity and nonspecific bindings were blocked by incubation with 3% hydrogen peroxide and non-immune goat serum, respectively. The sections were then incubated with goat anti-human IL-22 (dilution 1:50; R&D Systems) overnight at 4 °C, with a biotinylated mouse anti-goat secondary antibody for 30 min, and with peroxidase-conjugated streptavidin for 10 min. Finally, the sections were counterstained with Mayer's hematoxylin.

Data analysis

To investigate the genetic association between cases and controls, all statistical analyses were performed using the SAS/Genetics program (version 9.1, SAS Institute, Cary, NC, USA). The association between

genotypes and disease status were tested by Cochrane–Armitage trend test. To compensate accurately for testing this model, significance was also estimated against the empirical distribution of the statistic after performing 10 000 permutations of the case and control labels for each marker.

We also applied logistic regression models to test for the genotype–phenotype correlations. Odds ratio with 95% confidence interval for each genotype associated with gastric MALT lymphoma was determined. There were five significant SNPs of *IL-22*. Of these, four had very similar minor allele frequencies and odds ratios. In order to evaluate whether these four significant SNPs were located in same linkage disequilibrium block, this study assessed pairwise linkage disequilibrium structure among these SNPs by Haploview software (version 4.1) (Broad Institute, Cambridge, MA, USA). Furthermore, we used SAS/HAPLOTYPE procedure to test association between disease status and this haplotype block.

RESULTS

Baseline characteristics of the participants

The 68 lymphoma patients were classified according to Mussh-off's modification of the Ann Arbor staging system; 50 cases were classified as stage IE and 18 were classified as stage IIE. There was no statistical difference in the distribution of age (57.41 ± 15.0 vs 53.83 ± 8.54 years) and gender (male/female: 37/31 vs 73/67) between patients and controls. The rate of *H. pylori* infection was significantly higher in the patient group (64/68, 94.1%) than in the control group (68/140, 48.6%; $P < 0.001$).

Genetic polymorphisms in IL-22 associated with the risk of gastric MALT lymphoma

Among the 35 *IL-22* SNPs examined, 29 passed the quality control (that is, call rate > 0.9, minor allele frequency in controls > 0.05, departure from Hardy–Weinberg equilibrium $P < 0.001$ in the control sample) and were further tested. Allele frequencies between the controls and cases significantly differed in 5 out of these 29 SNPs, namely rs1179246 (A/C), rs2227485 (T/C), rs4913428 (C/A), rs1026788 (G/A) and rs7314777 (C/T) (Supplementary Appendix III). Four genotypes (rs1179246, rs2227485, rs4913428 rs1026788) were associated with an approximately threefold higher risk of gastric MALT lymphoma as compared with their homozygous counterpart. The TT genotype at rs7314777 was associated with an odds ratio of 9.88 (95% confidence interval, 1.29–75.63) as opposed to the CT genotype (Table 1).

Because four significant SNPs were located in the same linkage disequilibrium block (Figure 1, $r^2 = 0.99$), we examined the disease association of these haplotypes and found that the A-C-C-A haplotype was significantly associated with an increased risk of MALT lymphoma ($P < 0.0044$), whereas the C-A-T-G haplotype with a reduced risk ($P < 0.0082$) (Table 2). The haplotype of A-C-C-A was associated with increased risks of gastric MALT lymphoma with an odds ratio of 1.82 (95% confidence interval, 1.20–2.76) (Table 2).

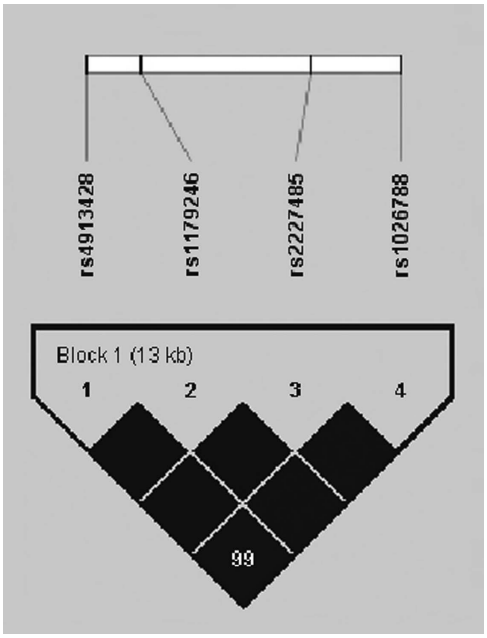

Figure 1. Linkage disequilibrium of *IL-22* polymorphisms. The four SNPs of *IL-22* (rs4913428, rs1179246, rs2227485 and rs1026788) are located in same linkage disequilibrium block ($r^2 = 0.99$).

Table 2. Association analysis of haplotypes consisting of rs4913428, rs1179246, rs2227485 and rs1026788 with response to gastric MALT lymphoma

Haplotype	*Haplotype frequency*				
	All (N = *208*)	*Ctrl* (N = *140*)	*Case* (N = *68*)	P-*value*[a]	*OR* *(95% CI)*[b]
A-C-C-A	0.488	0.439	0.588	0.0044	1.82 (1.20–2.76)
C-A-T-G	0.505	0.550	0.412	0.0082	0.57 (0.38–0.87)

Abbreviations: CI, confidence interval; Ctrl, control; MALT, mucosa-associated lymphoid tissue; OR, odds ratio;. [a]*P*-value was calculated by SAS/HAPLOTYPE as described in the Materials and methods. [b]Odds ratio of each haplotype was calculated as carriage versus noncarriage of the haplotype.

Table 1. Association study for the significant SNPs in *IL-22*

SNP	*Allelic OR (95% CI)*	$P_{allelic}$	*Genotype OR*			$P_{genotype}$
			00	*0X*	*XX*	
rs4913428	1.84 (1.21–2.80)	0.0045	1	1.57 (0.74–3.34)	2.98 (1.32–6.75)	0.0272
rs1179246	1.87 (1.23–2.84)	0.0037	1	1.63 (0.77–3.47)	3.05 (1.35–6.90)	0.0245
rs2227485	1.87 (1.23–2.84)	0.0037	1	1.63 (0.77–3.47)	3.05 (1.35–6.90)	0.0245
rs1026788	1.82 (1.20–2.78)	0.0052	1	1.69 (0.79–3.58)	2.85 (1.27–6.42)	0.0386
rs7314777	8.03 (1.06–61.07)	0.0441		1	9.88 (1.29–75.63)	0.0274

Abbreviations: CI, confidence interval; IL-22, interleukin-22; OR, odds ratio; SNP, single-nucleotide polymorphism. $P_{allelic}$ and allelic OR were calculated using logistic regression base on allelic model. $P_{genotype}$ and genotype OR were calculated using logistic regression based on genotype model. Genotype OR for heterozygous (0X) and homozygous carries (XX) compared with noncarriers (00).

Co-culture of PBMCs with *H. pylori* leads to secretion of IL-22 that induces expression of antimicrobial proteins in gastric epithelial cell lines

To understand the involvement of IL-22 in *H. pylori* infection, we first examined whether *H. pylori* was able to stimulate PBMCs to secrete IL-22. PBMCs co-cultured with *H. pylori* secreted significantly higher levels of IL-22 as compared with those without *H. pylori* co-culture ($P < 0.05$; Figure 2a). Because IL-22 is mainly produced by $CD4^+$ T cells,[26,27] we further examined whether the peripheral $CD4^+$ T cells co-cultured with *H. pylori* in the presence of antigen-presenting cells were able to induce IL-22 production. As expected, peripheral $CD4^+$ T cells, but not $CD8^+$ T cells, co-cultured with *H. pylori*-induced IL-22 secretion ($P < 0.05$; Figure 2b).

Given that IL-22 has been shown to stimulate the production of antimicrobial proteins,[28,29] we then investigated whether IL-22 was able to stimulate the human gastric epithelial cells (AGS cell lines) to produce antimicrobial proteins. Incubation of AGS cells with IL-22 at the concentration of 20 ng/ml significantly induced *RegIII*α and *lipocalin-2* (*LCN-2*) expression (Figure 3). The levels of *RegIII*α expression in cells stimulated with IL-22 for 6, 12 and 24 h were increased 2-, 5-, and 7-fold, respectively, as compared with the unstimulated cells at the corresponding time points. The levels of *lipocalin-2* expression were markedly increased 13-fold in cells stimulated with IL-22 for 24 h.

IL-22 polymorphisms seem not to be associated with the levels of IL-22 production

Knowing that five SNPs of *IL-22* were associated with susceptibility to MALT lymphoma, we next asked whether *IL-22* polymorphisms showed any functional relevance. We examined whether the number of risk alleles of *IL-22* polymorphisms might be associated with the levels of IL-22 production in PBMCs stimulated with *H. pylori*. We hypothesized that patients who have the highest number of risk allele of *IL-22* polymorphisms (10 risk alleles of 5 polymorphisms) should have the most impact on IL-22 production. We compared the levels of IL-22 production in *H. pylori*-stimulated PBMCs from patients who had the highest number of risk alleles of *IL-22* polymorphisms (10 risk alleles; CC at rs1179246, CC at rs2227485, AA at rs4913428, AA at rs1026788,and TT at rs7314777) and from patients who had the lowest risk alleles (2 risk alleles; TT at rs7314777). The co-culture of *H. pylori* with PBMCs obtained from patients with 10 risk or 2 risk alleles of *IL-22* polymorphisms significantly induced IL-22 production as compared with PBMCs without *H. pylori* stimulation. However, the levels of IL-22 produced by PBMCs co-cultured with *H. pylori* were not significantly different between patients with 10 risk alleles and those with 2 risk alleles, although IL-22 production seemed somewhat lower in patients with 10 risk alleles of *IL-22* polymorphisms (Figure 4).

Gastric tissue expression of IL-22 in relation to treatment responses

The expression of IL-22 in gastric tissues from 41 patients with *H. pylori*-positive stage IE/IIE1 low-grade gastric MALT lymphoma was evaluated by immunohistochemical staining. Among these patients, 22 responded to *H. pylori* eradication therapy and 19 did not (Table 3). Interestingly, expression of IL-22 (Figure 5) was significantly more prevalent in patients who responded to *H. pylori*

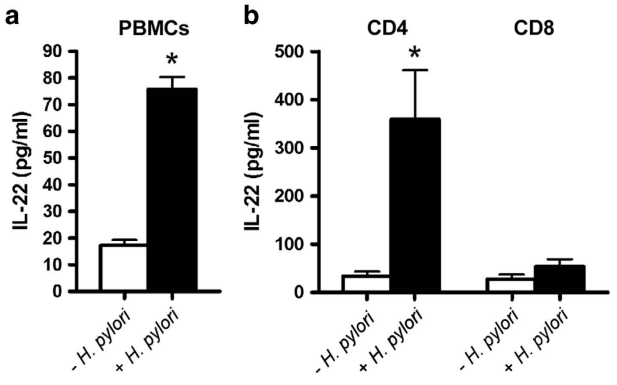

Figure 2. Stimulation of PBMCs or T cells with *H. pylori* leads to the production of IL-22. (**a**) PBMCs isolated from health donors ($n = 5$) were co-cultured with *H. pylori* (multiplicity of infection (MOI) = 30) for 48 h. The culture supernatants were collected and assayed for IL-22 levels by enzyme-linked immunosorbent assay (ELISA). Data are means ± s.e.m. from five subjects. $^*P < 0.05$ compared with PBMCs without *H. pylori* stimulation. (**b**) $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs as described in the Materials and methods. Isolated $CD4^+$ and $CD8^+$ T cells were co-cultured with *H. pylori* in the presence of antigen-presenting cells for 48 h. The culture supernatants were collected and subjected to the determination of IL-22 levels by ELISA. Data are means ± s.e.m. $^*P < 0.05$ compared with $CD4^+$ T cells without *H. pylori* stimulation.

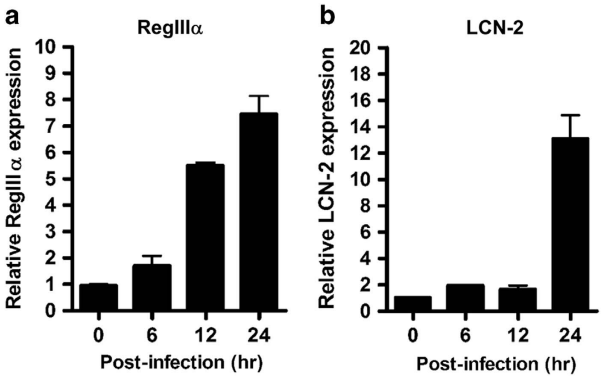

Figure 3. IL-22 induces the expression of antimicrobial proteins, RegIIIα and lipocalin-2, in AGS cells. AGS cells were cultured in the absence or presence of IL-22 (20 ng/ml) for the indicated times and the expression of RegIIIα (**a**) and lipocalin-2 (LCN-2) (**b**) in the cells was determined by real-time PCR. The relative expressions of RegIIIα and lipocalin-2 are expressed as fold over unstimulated cells. Data are means ± s.e.m. from two independent experiments.

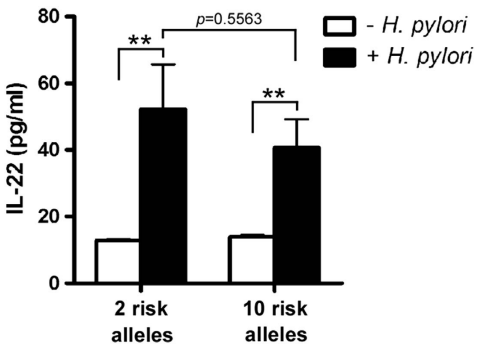

Figure 4. *IL-22* polymorphisms seem not to be associated with the levels of IL-22 production. The PBMCs isolated from patients with 10 risk alleles and 2 risk alleles of IL-22 polymorphism were subjected to the co-culture with *H. pylori* followed by the determination of IL-22 levels in culture supernatants as described in Figure 2a. $^*P < 0.01$ compared with PBMCs without *H. pylori* stimulation.

eradication (14/22, 63.6%) as compared with those who did not (4/19, 21.1%) ($P = 0.006$).

DISCUSSION

A growing body of evidence indicates that the interaction between *H. pylori* and host immune responses, in particular pro-inflammatory cytokines and chemokines, determines the pathogenesis of gastric MALT lymphomagenesis.[16,30,31] This study focused on the SNPs in genes encoding cytokines, chemokines and their receptors that were known to participate in gastrointestinal immune and inflammatory responses. For the first time, we demonstrated that the genetic polymorphism of *IL-22* was associated with risk of *H. pylori*-induced gastric MALT lymphoma. The C allele at rs1179246, C allele at rs2227485, A allele at rs4913428, A allele at rs1026788 and T allele at rs7314777 were significantly associated with increased risks of the disease. Our *in vitro* data further supported involvement of IL-22 in the gastric immune reactions against *H. pylori*. Moreover, the tissue expression pattern of IL-22 in gastric mucosa predicted treatment response to *H. pylori* eradication in patients with *H. pylori*-induced gastric MALT lymphoma. Collectively, these findings suggest that IL-22 plays a role in gastric MALT lymphoma induced by *H. pylori*.

Table 3. IL-22 expression predicted the treatment response to *H. pylori* eradication

	HPET responsive	*HPET unresponsive*
No IL-22 expression	8 (36%)	15 (79%)
IL-22 expression	14 (64%)	4 (21%)
	Chi-square test, $P = 0.006$	

Abbreviations: IL-22, interleukin-22; HPET, *Helicobacter pylori* eradication therapy.

Genetic factors conferring susceptibility to the development of gastric MALT lymphoma remain largely undetermined. Given that *H. pylori* infection induces Th1 and Th17 responses, and that Th1 and Th17 are major T-cell subsets producing IL-22 in humans,[26,27,32] the genetic variants in *IL-22* are plausible predisposing factors. IL-22 exerts its biological function through IL-22 receptor, a heterodimeric receptor complex composed of IL-22R1 and IL-10R2.[33–35] Of interest, IL-22 receptor (IL-22R1) is expressed exclusively in the skin, respiratory and digestive tissues,[36] implicating the pivotal role of IL-22 in innate mucosal immunity. Previous studies demonstrated that IL-22 was able to induce the synthesis of antimicrobial proteins in keratinocytes and epithelial cells of intestine and lung to mediate innate host defense against bacteria.[29,36,37] In line with these findings, our study demonstrated that IL-22 induces antimicrobial proteins, RegIIIα and lipocalin-2, in gastric epithelial cells. It is plausible that *H. pylori* stimulates immune cells to secrete IL-22 that then induces the expression of antimicrobials to protect epithelial cells from further infection by *H. pylori*. In this regard, IL-22 may have a protective role in gastric mucosa infected by *H. pylori*. Intriguingly, we discovered that the expression of IL-22 in gastric mucosa could serve as a predictor for therapeutic outcomes, although the underlying mechanism remained elusive.

Despite its importance in mediating host defense against bacterial infection, IL-22 may paradoxically lead to pathological inflammation and thus promote tumorigenesis. IL-22 signals through the Janus kinase/signal transducer and activator of transcription (STAT) signal pathway, and results in the activation of STAT3 through tyrosine phosphorylation.[38] Several studies have implicated IL-22 in the development of some types of lymphoma. For instance, IL22R1 is aberrantly expressed in anaplastic lymphoma kinase-positive anaplastic large-cell lymphoma that produces endogenous IL-22, forming an autocrine stimulatory

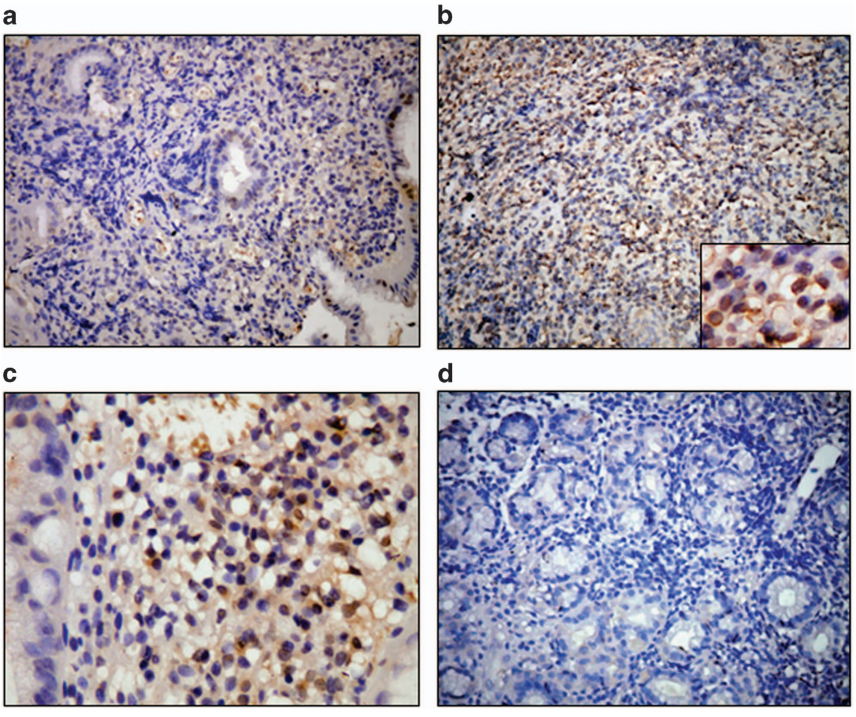

Figure 5. IL-22 expression in gastric MALT lymphoma tissues correlated with therapeutic response to *H. pylori* eradication therapy. Representative gastric mucosa tissues immunohistochemically staining for IL-22 with moderate expression (**a**) or strong expression (right bottom in (**b**), ×1000) (**b**, **c**) in patients whose tumor responded to *H. pylori* eradication, and those that did not respond (**d**).

loop to induce STAT3 activation and thus promoting tumorigenicity.[39,40] Similarly, IL-22R1 was also aberrantly expressed in mantle cell lymphoma, and stimulation of mantel cell lymphoma cell lines with recombinant IL-22 activates the signaling of STAT3, resulting in increased cell growth.[41] Altogether, these studies suggest that IL-22 signaling is functionally and biologically significant in lymphomagenesis. Given our findings that several genetic polymorphisms in *IL-22* were associated with increased risk of *H. pylori*-induced gastric MALT lymphoma, and that most *H. pylori*-induced gastric MALT lymphomas expressed IL-22, it would be interesting to investigate whether IL-22R1 is also expressed in *H. pylori*-induced gastric MALT lymphoma, and whether subsequent IL-22 signaling is able to promote the formation of gastric MALT lymphoma through the activation of STAT3.

Little is known about the clinical implication of *IL-22* polymorphisms. Hennig *et al.*[42] found that the T allele at rs1012356 and the C allele at rs1179251 were associated with effective viral clearance and improved response to antiviral treatment in chronic hepatitis C patients. Thompson *et al.*[43] discovered that the G variant at rs1179251 was associated with an increased risk of colorectal cancer. Zhang *et al.*[44] showed that the A allele at rs2227473 in the promoter region of *IL-22* was associated with decreased susceptibility to *Mycobacterium tuberculosis* infection. Our study adds one more disease of which susceptibility is influenced by *IL-22* genetic variation. We have tried to correlate the *IL-22* polymorphisms with the production of IL-22; however, no difference was found in IL-22 production in *H. pylori*-stimulated PBMCs from patients with 10 risk alleles of *IL-22* polymorphisms and from those with 2 risk alleles. Given that *H. pylori*-induced MALT lymphoma is a disease highly mediated by immune/inflammatory responses,[4,5,16] and that the microenvironment of the inflammatory tissues is conditioned by the interplays of many different types of cells, cytokines and chemokines, it is likely that the *in vitro* experiments may not directly reflect the physiological function *in vivo*. Notably, because the cytokine milieu plays an important role in the outcome of *H. pylori* infection, particularly cytokines involved in the innate immune responses, the genetic polymorphisms of those cytokines likely determine the susceptibility to the MALT lymphoma. Few studies have reported the genetic polymorphisms of cytokines contributing to the susceptibility to the MALT lymphoma. *TNF*–α (−857 T allele) has been reported to have a significantly increased risk to development of MALT lymphoma.[18,45] In addition, the genetic polymorphisms of *IL-1*β or *IL-10* have been shown in association with the susceptibility of MALT lymphoma in some ethnic background of studies.[46,47] Whether the genetic polymorphisms of *IL-10* or *IL-1*β are associated with MALT lymphoma in Han Chinese remains to be determined. It is plausible that the potential interaction of different cytokine gene variations is important in determining the susceptibility of gastric MALT lymphoma. Elucidating the biological implication underlying the genetic polymorphisms of these cytokines in gastric MALT lymphoma warrants further investigation.

Although there was a correlation between the expression of IL-22 in gastric tissues and the tumor's response to *H. pylori* eradication therapy, we found no association between the identified *IL-22* SNPs or haplotypes and the therapeutic response. It is possible that these susceptible genotypes influence only in the early phase of MALT lymphomagenesis but do not play significant roles in the fully developed MALT lymphoma. Given that the early phase of gastric MALT lymphomagenesis is characterized by long-term *H. pylori* stimulation, we hypothesize that during this stage infiltrating immune cells stimulated by *H. pylori* lead to IL-22 secretion to induce the production of antimicrobial proteins by gastric epithelial cells in order to eliminate the pathogen. It seems reasonable to assume that genetic polymorphisms in *IL-22* have a major impact on the early stage of pathogenesis. Nevertheless, the exact pathogenic mechanisms underlying this genetic susceptibility remain elusive.

In view of the rarity of gastric MALT lymphoma, the absolute risk would still appear low in individuals who carry the risk alleles of *IL-22*. Given that 0.01% of the *H. pylori*-infected patients eventually develop MALT lymphoma and that the genetic polymorphisms of *IL-22* we identified mounts to an approximately threefold higher risk, our findings suggest that the risk of developing this lymphoma would only be 0.03% in patients with the risk alleles. However, this magnitude of relative risk is commonly observed in a certain genetic predisposition to a complex disease. Besides, our study did not intend to cover all potential susceptible genes. Instead, with a rational candidate approach, we aimed to discover unreported genetic susceptibility and further explore its clinical relevance. In fact, we and others have identified several other genetic predispositions to the disease.[18–20,45–47] Moreover, it is possible that these identified genotypes are not pathogenic themselves, but simply markers in linkage disequilibrium with the causative variants. Further researches are warranted to elucidate how these genotypes affect the susceptibility to *H. pylori*-induced MALT lymphoma. Finally, as this study enrolled only Han Chinese participants, it would be interesting to know whether our findings are applicable to other ethnic populations.

In summary, this study demonstrates for the first time that IL-22 plays a role in the pathogenesis of *H. pylori*-induced gastric MALT lymphoma. We discovered that genetic polymorphism of *IL-22* was associated with the susceptibility to this rare lymphoma and that gastric tissues expressing IL-22 correlated with tumor response to *H. pylori* eradication. These findings will add to our understanding of the disease spectrum related to *H. pylori* infection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Guarantor of the article: Fang Liao and Ming-Shiang Wu; concept and design: Fang Liao, Yao-Chun Hsu and Ming-Shiang Wu; analysis and interpretation of the data: Fang Liao, Sung-Hsin Kuo, Ya-Chi Yang, Jia-Perng Chen, Cathy SJ Fann and Ming-Shiang Wu; collection and assembly of the data: all authors; drafting of the article: Fang Liao, Yao-Chun Hsu and Ming-Shiang Wu; critical revision: all authors; final approval: Fang Liao, Yao-Chun Hsu and Ming-Shiang Wu.

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