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Systemic Cytokine Levels and Subsequent Risk of Gastric Cancer in Chinese Women

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Abstract

Background—The control of the host cytokine network is known to influence gastric cancer susceptibility; the specific inflammatory responses in gastric carcinogenesis remain unclear.

Methods—We prospectively examined the relationships of plasma levels of interleukin (IL)-1β, IL6, IL8 and tumor necrosis factor (TNF)-α to gastric cancer risk within The Shanghai Women's Health Study. Two controls were matched to each case by age, menopausal status and sample collection parameters. The associations of gastric cancer risk and tertile of cytokine levels were estimated by odds ratios (ORs) and 95 per cent confidence intervals (% CIs) from conditional logistic regression, adjusting for education.

Results—During a median follow-up period of 4 years (range: 0.1–8), 141 women developed gastric cancer and were matched to 282 cancer-free study participants. Elevated levels of plasma IL6 were associated with an increased risk of gastric cancer (P_{trend}=0.04). Risk increased 70% (OR=1.7, 95% CI, 1.0, 3.0) for women in the highest tertile (> 4 pg/mL) of IL-6 compared to those in the lowest tertile (<1.8 pg/mL). The association with IL6 was stronger after 4 years of follow-up (OR=2.6, 95% CI, 1.0, 6.7 for highest vs. lowest tertile) compared to an OR of 1.4 (0.7, 2.9) for those diagnosed within 1–4 years of follow-up. No associations were observed with the other examined pro-inflammatory cytokines, IL1β, IL8 and TNFα.

Conclusions—Systemic plasma IL6 levels may inform long-term gastric cancer risk. This novel finding awaits confirmation in future studies with sequential plasma collection.

Keywords

cytokine; gastric cancer; prediagnostic

Introduction

Gastric cancer has the second highest cancer mortality globally(1). A strong risk factor for gastric cancer is persistent colonization in the stomach lining by the Class 1 gastric carcinogen, *Helicobacter pylori*(2). *H. pylori* chronically infects half of the global human

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population(3). Whereas chronic *H. pylori* infection induces specific local and systemic inflammatory responses resulting in gastritis and inflammation-related comorbidities, only a small percentage of infected persons develop gastric cancer (4, 5).

Bacterial and human host factors modulate the risk of gastric cancer development. In terms of the bacterial factors, the virulence of the *H. pylori* can be identified by presence of a 40-kilo basepairs region of chromosomal DNA termed as cytotoxin-associated (*cag*) pathogenicity island (cag^+ *versus* cag^-). Persons infected with the cag^+ strain have a more pronounced inflammatory response as well as a higher risk for gastric adenocarcinoma as compared to those carrying the cag^- strains of *H. pylori*. However, a large proportion of hosts colonized by the cag^+ strain remain asymptomatic(6). The key players in human host response that underlie *H. pylori*-associated gastric carcinogenesis remain unclear.

In vitro and *in vivo* studies have evidenced the role of cytokines, a key regulator of immune system, in gastric carcinogenesis. We and others have shown that one of the human host factors associated with gastric cancer susceptibility are polymorphisms in genes encoding pro-inflammatory cytokines(7–9) that may act as proxies for the strength of inflammatory responses to *H. pylori* infection. Based on *in vitro* studies of *H. pylori* persistence as well as comparison studies of cytokine levels between gastric cancer patients and controls, we hypothesize that higher systemic levels of certain interleukins(IL) may increase gastric cancer risk. We investigate the relationships between pre-diagnostic IL-1β, IL6, IL-10, Tumor Necrosis Factor(TNF)-α and gastric cancer risk in a nested case-control study within the Shanghai Women Health Study (SWHS), a population-based cohort in Shanghai (10).

Methods and Materials

Study population

The study population is nested within the Shanghai Women Health Study (SWHS), a population-based prospective cohort as previously described(10). Briefly, 81,170 women aged 40 to 70 years residing in seven urban communities in Shanghai, China, during March 1997 and May 2000 were invited to participate in the study. Of these, 74,942 women were interviewed, resulting in a response rate of 92.3%. At recruitment, information on lifestyle factors, anthropometric measurements and biospecimens were collected. Incident gastric cancer cases among cohort members were identified through active biennial follow-ups and record linkage with the population-based Shanghai Cancer Registry, the Shanghai Vital Statistics Unit and Shanghai Resident Registry. The Institutional Review Boards at the Shanghai Cancer Institute (People's Republic of China), National Cancer Institute (Bethesda, MD) and Vanderbilt University (Nashville, TN) approved this study.

Nested Case-Control

Cases—Of 74,942 women in the cohort, 56,832 provided a blood sample. Exclusion criteria were previous cancer reported at the baseline interview or cancer diagnosis within a month from the date of sample collection. As of December 2006, 141 gastric cancer cases (International Classification of Disease version 9 codes of 151.0–151.9) were newly diagnosed among cohort members. Of these cases, 13 (9%) were classified as gastric cardiac cancers.

Controls—Two controls per case, resulting in a total of 282 controls, were randomly sampled without replacement from the cohort and matched to the case on the following variables at sample collection: menopausal status, date (<1 month) and time of sample collection (morning/afternoon), age (<2 years), time interval since last meal (< 2 hours), and availability of plasma samples.

Questionnaire data and non-cytokine serological markers—Fruit and vegetable intake was quantified by trained interviewers using a comprehensive food frequency questionnaire representing 90% of foods consumed in Shanghai in 1996. Smokers were classified as subjects who smoked at least one cigarette per day for more than six months. Alcohol drinkers were those who drank alcohol more than three times a week for six months. Self-reported aspirin/non-steroidal anti-inflammatory drugs (NSAIDs) use and frequency during the past year was obtained at baseline. Self-reported medication use during the past 7 days and the past 24 hours was obtained at blood collection.

H. pylori infection and *cag* seropositivity was examined as presence of IgG antibodies against whole cell or the *CagA* H. pylori antigens using ELISA (*H. pylori* Biohit ELISA kit, Biohit Plc, Finland) and immunoblot (Helocoblot 2.0, Geneslab Diagnostics, Singapore) assays, respectively.

Cytokine measurements—Plasma were processed from a 10-ml blood sample collected in a ethylenediaminetetraacetic acid Vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, New Jersey), kept at 0–4°C no longer than 6 hours prior to processing and stored at −70°C. IL1β, IL6, IL10 and TNFα were measured simultaneously in 25μl plasma using high-sensitivity LINCO*plex* kit (Luminex xMAP Technology) at the Vanderbilt Hormone Assay & Analytical Services Core. Sensitivities of the IL1β, IL6, IL10 and TNFα assays according to manufacturer protocol were 0.1 pg/mL, 1.6 pg/mL, 0.2 pg/mL and 0.14 pg/mL, respectively.

The absolute concentration of each cytokine (pg/mL) in each sample was determined by relating the fluorescence intensity to a standard curve fitted by a 4-point-regression. Cytokine measurements were carried out in duplicates. Intra-assay variability was assessed with 19 replicate/duplicates of quality control samples within a single assay/plate of 38 samples. Interassay variability across 12 assays was assessed by using the mean and standard deviation of two replicate samples each in twelve separate assays. The intraassay coefficient of variation ranged from 3% to 17%; inter-assay CVs ranged from 9% to 20%.

Statistical Methods

To accommodate the matched design, we fit conditional logistic regression (CLR) models to estimate odds ratios (ORs) and 95% CI for the association of gastric cancer risk with IL1β, IL6, IL10 and TNFα, respectively. Models were additionally adjusted for education level (continuous). Covariates considered as potential confounders were self-reported antibiotic use within 1 year of blood collection, self-reported NSAIDs use and frequency during the past year, body mass index, and smoking history. None were included in the final model because they did not substantially alter the parameter estimates for the exposures (plasma cytokine levels).

Plasma cytokine levels were classified into tertiles based on the distribution among controls. Using the lowest tertile as reference, we assessed ORs for the second, and third tertiles. Measurements below detection limits (assay detection limits (pg/mL): IL1β, 0.06; IL6, 0.1; IL8, 0.11; TNFα, 0.05) were included in the lowest tertile. To assess linear trends in risk, ascending tertiles of cytokine levels were treated as ordered categories.

Stratum-specific ORs of each cytokine and gastric cancer risk were estimated, respectively, for early (i.e., case diagnosed 1–4 years from baseline and corresponding controls) or late duration of follow-up (i.e., > 4 years). Formal test of heterogeneity were conducted by comparing stratum-specific p-values for additive models using a one-degree of freedom Wald test. Sixteen cases diagnosed within 1 year from blood collection were excluded in the

stratum-specific analyses; inclusion of cases diagnosed less than a year of blood collection did not significantly alter the parameter estimates (data not shown).

All p-values are two-sided and statistical analyses were performed using STATA 10.0 (Stata Corp, College Station, TX).

Results

The median duration between blood sample collection and cancer diagnosis was 3.75 years (range: 0.12, 8.39). *H. pylori* prevalence was high (92% in controls, Table 1). Specifically, 92% of the controls were positive for H. pylori; 89 % were seropositive for CagA strains. By virtue of matching, cases and controls had similar distributions of age at interview/ recruitment (median: 61 years) and menopausal status (post-menopausal: 79%). Cases and controls did not differ significantly by education level, alcohol intake, smoking history, fruits and vegetables intake, or *H. pylori* seropositivity (Table 1). Cases had a lower frequency of self-reported antibiotic use within 1 year of blood collection.

Table 2 summarizes the association of plasma level of each cytokine and gastric cancer risk. One case and three controls for IL1β and one control for IL8 were below detection limits of the assay. Women in the highest tertile (> 4 pg/mL) of IL6 were at increased risk of gastric cancer (OR=1.7, 95% CI, 1.0, 3.0) as compared to those in the lowest tertile (<1.8 pg/mL). Similar results were observed when restricted to women who developed gastric cancer one or more years after plasma collection, and/or after adjusted for medication use within one week before plasma collection (data not shown). Exclusion of thirteen gastric cardia cancer cases produced similar results (e.g., lowest *versus* highest tertile of plasma IL6 levels: OR=2.0, 95% CI, 1.1, 3.8; data not presented in tabular form). Analyses restricted to subjects seropositive for *H. pylori* infection yield similar results.

The magnitude of association with IL6 increased with duration of follow-up. The OR comparing the highest versus the lowest tertile of IL6 was 2.6 (1.0, 6.7) after >4 years of follow-up compared to an OR of 1.4 (0.7, 2.9) for those diagnosed during 1–4 years of follow-up (Table 3). The p for heterogeneity, however, was not statistically significant.

No statistically significant associations were observed with the other examined pro-inflammatory cytokines, IL1β, IL8 and TNFα (Tables 2 and 3).

Discussion

Persistent *H. pylori* infection is associated with systemic immune response(11) that possibly contribute to the development of gastric adenocarcinoma. Our results suggest that systemic plasma IL6 levels may increase subsequent gastric cancer risk. Our findings could be suggestive of the concept that systemic IL6 levels may be a biomarker for low grade inflammation. Soluble inflammatory markers can stratify populations into groups that may benefit from intervention and these markers themselves are possible intervention targets. To our knowledge, this is the first report that demonstrates elevated IL6 levels four or more years prior to cancer diagnosis is a risk factor for gastric cancer. Further, consistent with *in vitro* and *in vivo* data of cytokines and numerous cancers, this is the first report of an association between prediagnostic systemic levels of cytokines with any cancer. The association between plasma IL6 levels and gastric cancer risk is consistent with the model where H. pylori infection induces a cascade of inflammatory reaction in gastric mucosa that leads to gastric atrophy and carcinogenesis. In humans, proinflammatory cytokines are known to accompany acute *H. pylori* infection. Expression levels of IL1β, IL8, and IL6 levels in the gastric biopsies of 20 human volunteers after 2 weeks experimentally infected with 10^4 to 10^{10} colony forming unit of *H. pylori* (12). Little is known about the key players

of chronic inflammation in gastric carcinogenesis. Our findings implicate the pertinent players in the host response as specific members of a class of regulators of the immune system called cytokines, specifically IL6 in gastric cancer susceptibility. Our findings are consistent with the mouse model of IL6 superfamily and gastric carcinogenesis(13). Chronic *H. pylori* infection induces chronic inflammation that includes a strong T-helper 1 cell type immune response, and thereby boosts the production of the IL6 family members and induces the subsequent downstream effects of disruption of the STAT1/3 and SHP2-Ras-ERK signaling pathways that leads to gastric hyperplasia(13). However, the findings of our study require careful interpretation as discussed further below.

In case-control and cross-sectional studies, IL6 levels are elevated in the sera and plasma of individuals with gastric cancer (14–17) as well as other inflammation-related cancers including colon(17–19) and prostate(20–22). However, cancer cells and the surrounding microenvironment secrete inflammatory markers including cytokines(23). Our study design minimizes the possibility that the high cytokine levels we observed are a consequence of cancer. To exclude the possibility that our findings may be due to the presence of undetected gastric cancer or early gastric cancer, we assessed the associations by restricting the analyses to those whose cancer was diagnosed after more than a year of follow-up. In addition, we stratified the associations between each cytokine and gastric cancer risk by follow up time. Assuming random sampling, these two groups may be a crude representation the average cytokine levels according to duration time and may approximate repeated measures in a longitudinal study. Risk associated with IL6 increased further with longer duration of follow-up, suggesting that this association is unlikely due to reverse causation. However, this novel finding awaits confirmation in other prospective studies with sequential plasma collection.

A limitation to our findings is the possibility of a type 1 error due to our small sample size. A potential weakness of our findings is that the increased risk of gastric cancer was observed only with a threshold of higher IL6 levels. We also explored non-linear associations between cytokine levels as well as latent factors of the 4 cytokines via principal components analyses; these alternative analyses did not yield additional insights. Similar statistically significant associations were not observed with the other three proinflammatory cytokines (IL1β, IL8 and TNFα). In our study, plasma IL6 levels in healthy women (controls) correlated with IL8 but not with IL1β and TNFα. These correlations are consistent with those observed in 60 Japanese gastric cancer patients(24). Though the *in vitro* evidence of the role of these four cytokines in gastric carcinogenesis were approximately similar, limited data on cross-sectional studies indicated that pre-operative circulating IL1β(15) and IL6(14–16) levels are elevated whereas TNFα levels are reduced(15) in gastric cancer patients as compared to non-diseased individuals. In our study, the associations between IL8 and TNFα levels in relation to gastric cancer risk were not statistically significant; however, the estimates appear to be consistent with the cross-sectional studies. The associations between IL1β and gastric cancer risk, while not statistically significant, were not consistent with the cross-sectional studies. However, the measurement error for plasma IL1β levels were much higher (CVs ~ 20%) than the other three cytokines (CVs < 10%). In addition, the volatility of the cytokines levels due to external stimuli may differ, for example, the induction of IL1β and TNFα levels are influenced by the exercise and circadian rhythm whereas IL6 levels are not affected(25). Another limitation of our study is that plasma cytokine levels may not represent robust markers of inflammation in the gastric lining. Although we have accounted for known stimuli, *e.g.*, smoking, obesity and anti-inflammation therapy, in our study design and/or statistical analyses, we did not exclude certain possibly common non-specific chronic inflammation conditions that drive systemic cytokine levels, *e.g.*, urinary tract infections(26) or chronic respiratory infections.

Our study has several strengths to address the hypothesis that pro-inflammatory cytokines predict gastric cancer risk. We capitalized on the prospective nature of our study design to minimize temporal ambiguity in the interpretation of our findings. In our high-risk population of more than 90% infected with *H. pylori*, we minimize confounding by non-specific *H. pylori*-related inflammation. In addition, the prevalence of other strong and common non-specific inflammatory stimulus is low in our population: tobacco use (2.4%), obesity (5.1%) and anti-inflammatory medication intake (NSAIDs intake, Table 1). In terms of measurement error, we also used sensitive and quantitative methods to measure the low levels of plasma cytokines(27, 28).

Using gastric cancer as a model of inflammation and cancer, our approach of prospectively examining soluble inflammatory markers in relation to cancer susceptibility has potential to elucidate the key players underlying the etiology of inflammation-related cancers.

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Abbreviations

H. pylori	Helicobacter pylori
IL-	Interleukin-
TNF-	Tumor Necrosis Factor-

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Table 1

Selected Characteristics of the Gastric Cases and Controls, Shanghai Women Health Study

Characteristics	Controls (N(%)) N=282	Cases (N(%)) N=141	Exact P-value
Educational Level			
College or higher	28 (9.9)	8 (5.8)	0.30
High school	59 (20.9)	26 (18.4)	
Middle school	82 (29.1)	39 (27.6)	
Elementary/less	113 (40.1)	68 (48.2)	
Alcohol intake			
Ever	5 (1.8)	2 (1.4)	1.0
Never	277 (98.2)	139 (98.6)	
Smoking			
Ever	13 (4.6)	9 (6.2)	0.49
Never	269 (95.4)	132 (93.8)	
Fruit and Vegetables Intake (serving/week)			
(median(range))	21 (6,35)	21 (7,28)	0.62
Antibiotic use within 1 year of blood collection			
No	256 (90.8)	136 (96.5)	0.05
Yes	26 (9.2)	5 (3.5)	
H. pylori seropositivity			
No	22 (7.8)	5 (3.5)	
Yes	260 (92.2)	136 (96.5)	0.14

Age at interview/recruitment (median:61 years) and menopausal status (post-menopausal: 79%) were the matching criteria for cases and controls. Fisher exact test or Kruskal-Wallis.

Table 2

Odds ratios and 95% confidence intervals for prediagnostic circulating Interleukin-1β, Interleukin-6, Interleukin-8 and Tumor Necrosis Factor-α and Gastric Cancer Risk in Shanghai Women.

Cytokines (pg/mL)	Controls/Case N=282/141	OR (95% CI)	P-value
Interleukin-1β			
<0.69	96/51	Reference	
0.70-1.55	94/45	0.85 (0.52–1.39)	
>1.56	92/45	0.87 (0.50–1.56)	0.60
Interleukin-6			
<1.76	95/40	Reference	
1.77-4.05	94/41	1.07 (0.61–1.87)	
>4.06	93/60	1.73 (1.00–3.00)	0.04
Interleukin-8			
<2.64	94/41	Reference	
2.65–7.16	94/47	1.12 (0.67–1.87)	
>7.17	94/53	1.41 (0.85–2.36)	0.18
Tumour Necrosis Factor-α			
<4.86	94/56	Reference	
4.87–7.16	94/42	0.68 (0.41–1.15)	
>7.17	94/43	0.74(0.42–1.30)	0.27

Estimates from conditional logistic regression, matched on age difference at sample collection(<2 years), menopausal status at sample collection, time of sample collection (morning/afternoon), date of sample collection (<1 month) and time interval since last meal (< 2 hours) and, adjusted for education level (continuous).

Assay detection limits (pg/mL): IL1β, 0.06; IL-6, 0.1; IL-8, 0.11; TNF, 0.05 Measurements under detection limits were included in the lowest tertile (IL1β: 1 case and 3 controls; IL8 -1 control).

Table 3

Odds ratios and 95% confidence intervals for pre-diagnostic circulating Interleukin-1β, Interleukin-6, Interleukin-8 and Tumor Necrosis Factor-α and Gastric Cancer Risk by follow-up time in Shanghai Women.

Cytokines (pg/mL)	1–4 years follow-up time		>4 years follow-up time	
	Controls/ Case	OR (95% CI)	Controls/ Case	OR (95% CI)
Interleukin-1β				
Low	44/20	Reference	43/21	Reference
Medium	41/28	1.41 (0.71, 2.80)	40/15	0.74(0.33,1.68)
High	37/13	0.62(0.25,1.56)	45/28	1.58(0.64,3.88)
		(P_{trend}=0.55)		(P_{trend}=0.40)
Interleukin-6				
Low	45/21	Reference	37/15	Reference
Medium	35/14	0.85 (0.38, 1.89)	49/20	1.19 (0.48,2.95)
High	42/26	1.37 (0.66,2.86)	42/29	2.56(0.98,6.71)
		(P_{trend}=0.41)		(P_{trend}=0.04)
Interleukin-8				
Low	43/20	Reference	37/16	Reference
Medium	43/24	1.21 (0.58, 2.54)	39/21	1.15 (0.52,2.55)
High	362/17	1.07(0.49,2.33)	52/27	1.44(0.65,3.21)
		(P_{trend}=0.88)		(P_{trend}=0.36)
Tumor Necrosis Factor-α				
Low	49/28	Reference	37/19	Reference
Medium	37/17	0.74 (0.34, 1.63)	42/24	1.07 (0.48,2.95)
High	36/16	0.70 (0.29,1.70)	49/21	0.95(0.39,2.26)
		(P_{trend}=0.41)		(P_{trend}=0.90)