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The Association of Circulating Inflammation Proteins and Gallstone Disease

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Abstract

Background: Inflammation plays a role in the development of both gallstones and gallbladder cancer; however, few studies have investigated the association of circulating inflammation proteins with risk of gallstones.

Methods: We measured 13 cytokines (including 10 interleukins), that have been associated with cancer in serum samples collected from 150 gallstone patients and 149 population-based controls from Shanghai, China, in 1997–2001. We estimated the associations of each cytokine, categorized into quartiles and coded as a trend, with risk of gallstones using logistic regression models adjusted for potential confounders.

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Results: Higher levels of interleukin (IL)-6, IL-10, IL-12 (p70), and IL-13 were associated with increased risk of gallstones (i.e., $P_{trend} < 0.003$, Bonferroni corrected), with odds ratios (ORs) that ranged from OR $_{\text{highest quartile [Q4] vs lowest quartile [Q1]}} = 3.2$ (95% confidence interval [CI]: 1.4, 7.5) for IL-13 to OR $_{\text{Q4 vs. Q1}} = 5.7$ (95% CI: 2.5, 13.5) for IL-12 (p70). In a regression model including all four interleukins, only IL-12 retained statistical significance ($P < 0.05$).

Conclusions: We found four circulating interleukins that were associated with gallstones. Future studies are needed to validate the findings and evaluate the common pathway or mechanism in the development of gallbladder diseases associated with these cytokine signatures.

Keywords

case-control study; cytokines; gallstones; inflammation; interleukins; risk factor

Introduction

Gallstone disease is prevalent in the general population, affecting 5% to 15% of adults worldwide [1–4]. The incidence has increased in recent years, particularly in Western populations [5–9]. The cholesterol stones comprise the vast majority (over 70%) of gallstones in Western countries, while both cholesterol stones and pigment stones are common in Asian countries [9–11]. Gallstones can result in several clinical diseases with severe symptoms, including cholecystitis, cholangitis, and acute pancreatitis. Importantly, gallstones play a key role in the development of gallbladder cancer, as suggested by the fact that approximately 90% or more of patients with gallbladder cancer have a history of gallstones [12, 13], with an odds ratio (OR) of >20 [12]. Both genetic and environmental factors may contribute to the formation of gallstones, and risk factors that contribute to the formation of stones vary across types [14]. For example, cholesterol stones are more likely to be associated with obesity, which may affect the concentration of the cholesterol level in bile, while pigment stones are more likely to be associated with biliary infections and hemochromatosis [14]. For example, *Clonorchis sinensis* infection is associated with the development of pigment stones [15]. In addition, inflammation has been proposed to play an essential role in gallstone development, as indicated by the fact that formation of cholesterol gallstones is preceded by histopathologic alterations in the gallbladder wall that indicate inflammation in both animal models and humans [16–18]. Some inflammation-related conditions, such as obesity, diabetes, and infections (e.g. *Helicobacter pylori*), are also associated with risk of cholesterol gallstones[19–21]. However, few studies have investigated the association between inflammation proteins and risk of gallstones [22].

Previously we showed that circulating inflammation proteins are significantly correlated with inflammation proteins measured in bile [23], suggesting that we can use blood samples to better understand the association between inflammation and gallstone risk. In addition, we found 26 inflammation proteins associated with gallbladder cancer risk when comparing levels of 49 inflammation-related proteins in serum from gallbladder cancer cases to those from patients with gallstones [23, 24]. Seven of the markers were associated with risk of early stage gallbladder cancer, suggesting that measurement of inflammation proteins may have clinical utility. This utility may extend to other gallbladder diseases, including gallstones. To better understand the role of inflammation in development of gallstones, we undertook an

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analysis of circulating cytokines and gallstone risk in a population-based case-control study in Shanghai, China.

Methods

Study population

The current study is based on biospecimens collected from the Shanghai Biliary Cancer Study, which has been previously described [12, 23]. In brief, between 1997 and 2001, a total of 368 patients with gallbladder cancer were enrolled; 774 patients with gallstones (>95% of the eligible gallstone cases) who were undergoing cholecystectomy or medical treatment at the same hospitals as the cancer cases were frequency matched by sex and age (in 5-year category) to the cases; 959 healthy controls (>83% of eligible controls) were also randomly selected from the Shanghai population and frequency matched to cancer cases on age and sex. Although we do not have information on whether the gallstone patients had active cholecystitis, we expect that the presence of severe active cholecystitis is low because cholecystectomy is not recommended for such patients.[25, 26] Gallstone status was confirmed through self-report of a gallstone history, operative reports and imaging data for cases with gallstones and 87% of the participating controls. In addition to a self-reported gallstone history, transabdominal ultrasound was performed to validate gallstone status and to identify silent gallstones. The Shanghai Cancer Institute and National Cancer Institute institutional review boards approved the study. Written informed consent was obtained for all participants.

We evaluated circulating inflammation proteins in 150 randomly selected gallstone patients who either had serum collected prior to surgery or did not have surgery, and 150 randomly selected healthy controls frequency matched by sex and age (in 5-year category, range: 35–74 years) to the gallstone cases. All gallstones were in gallbladder (N= 126), gallbladder neck (N=13), or both (N=11). A total of 121 gallstones were cholesterol/mixed stones, 16 were pigment/black stones, and 13 had unknown composition. None of the controls had silent gallstones. One control did not have sufficient serum for this assay. We used the Milliplex high-sensitivity (EMD Millipore, Billerica, MA) kit to test for 16 cytokines that have been associated with cancer (interleukin [IL]-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12[p70], IL-13, IL-17α, IL-21, IL-23, CCL-3 [CC-Chemokine Ligand 3], CCL-20, GM-CSF [Granulocyte macrophage-colony stimulating factor], and CX3CL1 [chemokine (C-X3-C motif) ligand 1]) [27]. Serum samples were incubated with beads in 96-well plates, after which fluorescently labeled detection antibodies were added. A Bio-Plex instrument and Bio-Plex Manager 6.1 software (Bio-Rad, Hercules, CA, USA) were used to analyze the 96-well plates

Details on quality controls have been previously published [23, 28, 29]. In brief, all samples were measured in a single batch, and blinded duplicate aliquots from 10 participants were selected to estimate the reproducibility of the assays. We calculated log-adjusted coefficients of variation (CVs) and intraclass correlation coefficients (ICCs) using a log-transformed general linear model. We excluded 3 proteins (CX3CL1, IL-5, and IL-21) with overall CVs > 30%, and/or ICCs <0.75. Thus, the final analysis included 13 evaluable cytokines including 10 interleukins, in 150 patients with gallstones and 149 controls.

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Statistical Analyses

All proteins were detectable in ≥75% of healthy controls and gallstone cases. We created four categories based on quartiles of values above the lower limit of quantitation using the distribution among healthy controls (subjects with undetectable values were included in the lowest quartile). These proteins were modeled both as categorical and ordinal variables (coded as 1, 2, 3, 4) to evaluate linear trend.

We used unconditional logistic regression models to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for associations of each cytokine individually with gallstones. Minimal adjustments for the models included the frequency-matching variables, age group at diagnosis/selection in categories (≤54, 55–65, or ≥66 years), and sex. Identification of potential confounders was based on prior knowledge. Fully adjusted models additionally included education (none/primary, junior middle, senior middle, some college), ever drinking, ever smoking, self-reported body mass index (underweight, <18.5; normal weight, 18.5–24.9; overweight, 25–29.9; obese, ≥ 30 kg/m^2), diabetes, and history of cholecystitis. Two-sided *P* values for trend across protein categories were computed using Wald tests. We tested for modification of cytokine associations by sex (which may differ in the prevalence of some lifestyle factors, such as smoking), BMI, and diabetes (both of which have been shown to be slightly higher among gallstone cases than controls) using likelihood ratio tests for nested models with and without interaction terms.

Statistical analyses were performed using SAS, version 9.4 (SAS Institute, Cary, NC, USA). The statistical tests were two sided with $\alpha = 0.05$. Bonferroni corrected *P* values (0.05/13=0.004) were calculated to account for multiple testing.

Results

Characteristics of the 150 cases with gallstones and the 149 population-based healthy controls, stratified by sex, are shown in Table 1. As expected, gallstone cases were more likely to report a history of cholecystitis than controls ($P < 0.001$ for both women and men), and women from the case group were more likely to be less educated than women from the control group ($P = 0.018$). Otherwise, we found no significant differences between the distribution of characteristics of cases and controls, stratified by sex.

Of the 13 cytokines, four were statistically significantly associated with gallstone risk in age- and sex- adjusted models and fully adjusted models (using P_{trend} <0.003, Bonferroni correction, Table 2). Higher levels of interleukin IL-6, IL-10, IL-12 (p70), and IL-13 were associated with a higher risk of gallstones, with odds rations ranging from $OR_{\text{highest quartile [Q4] vs lowest quartile [Q1]}} = 3.2$ (95% CI: 1.4, 7.5) for IL-13 to $OR_{Q4 \text{ vs. } Q1} = 5.7$ (95% CI: 2.5, 13.5) for IL-12 (p70) in fully adjusted models. The association between the remaining nine markers (IL-1β, IL-4, IL-7, IL-8, IL-17α, IL-23, CCL3, CCL20, and GM-CSF) and gallstone risk are presented in Supplementary Table 1). Of the 4 proteins significantly associated with gallstone risk after Bonferroni correction, the odds ratios did not differ statistically by sex, BMI, or history of diabetes, although power was limited for these analyses (all P for heterogeneity >0.05, Supplementary Table 2).

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Among controls, the correlations between the 4 proteins significantly associated with gallstone risk after Bonferroni correction were strong (spearman correlation coefficient range: 0.314 – 0.490; all P <0.0001,). In a regression model including these 4 proteins, only IL-12 retained statistical significance (P < 0.05).

Discussion

The present study shows associations between gallstones and circulating IL-6, IL-10, IL-12 (p70), and IL-13. In particular, IL-12 (p70) was independently associated with risk of gallstones. Thus, prospective studies of IL-12 (p70) and gallstone risk may be warranted to confirm these findings.

Our results are in line with a previous study indicating that inflammation, as measured by a few cytokines (i.e., IL-1α, IL-6, IL-8, and tumor necrosis factor [TNF]-α), is associated with risk of gallstone diseases [22]. However, the magnitude of associations between cytokines with risk of gallstones was much smaller than the associations of cytokines with risk of gallbladder cancer [23]. For example, our previous study found an $OR_{Q4\ vs.\ Q1}$ of IL-8 with gallbladder cancer versus gallstones of 96.8 (95% CI: 11.9, 790.2),[23] whereas no significant association was observed between IL-8 and gallstones in the present study. In addition, the magnitude of associations between gallbladder cancer and gallstones for IL-6 ($OR_{Q4\ vs.\ Q1}$: = 21.6, 95% CI: 8.0, 58.6) and IL-10 ($OR_{Q4\ vs.\ Q1}$: = 6.1, 95% CI: 2.6, 14.3) (unpublished data) is much higher than that between gallstones and healthy controls. These findings suggest that gallstones do not cause changes in the circulating inflammation profile that are as marked as those caused by gallbladder cancer. If true, there may be strong potential for inflammation proteins to stratify patients with gallstones based on risk for gallbladder cancer [24].

Although the role of cytokines in the gallbladder and their influence on gallstone formation is not clear, it is well established that the formation of gallstone involves inflammation [18, 30]. For example, inflammation may alter the metabolism of several proteins and lipids; these changes may alter cholesterol and bile acid metabolism and increase bile salt levels, thus causing formation of gallstones. In the present study, four cytokines (IL-6, IL-10, IL-12 [p70] and IL-13) were associated with gallstones. These observations are biologically plausible. For example, IL-6 can be expressed by biliary epithelium, and it is known to have proliferative effects on several nonhematopoietic cells [17, 31]. IL-6 can also cause infiltration of inflammatory cells and increase gallbladder wall thickness [17, 31]. To our knowledge, no previous studies have investigated the role of IL-10, IL-12, and IL-13 on the formation of gallstones. Studies have shown that IL-10 predominantly inhibits lipopolysaccharide (LPS) and bacterial product-mediated induction of pro-inflammatory cytokines, including IL-12 [32]. IL-12 can stimulate the production of TNF-α from T cells and natural killer cells, which directly affects the absorption, secretion, and functions of gallbladder epithelial cells [16]. Interleukin-13 can control IgE synthesis, which causes allergic inflammation and different diseases including asthma [33]; therefore, the association between asthma and gallstones merits further investigation. In an adjusted model including all four proteins, only IL-12 remained statistically significant. Although our results are cross-sectional, this finding suggests that the role of IL-12 in the formation of gallstones merits future investigation. It is

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noteworthy that IL-10, IL-12, and IL-13 were significantly associated gallstones, but not with gallbladder cancer [24], suggesting that the inflammatory processes that drive the development of gallstones may differ from those that drive the development of gallbladder cancer.

The present study is one of the largest studies of cytokines and gallstone to date. Misclassification of disease status was minimized through comprehensive pathology and clinical review, which provided nearly complete confirmation of case status. However, our results should be interpreted in light of several limitations. First, although there is strong biologic plausibility for the association of cytokines with gallstone risk, our observations require replication. Second, reverse causation cannot be ruled out due to the cross-sectional case-control design (i.e., inflammation protein levels may be elevated due to the presence of gallstones or acute cholecystitis among a minority of cases). However, while histologic evidence of cholecystitis was seen among 145 of 147 cases (99%) reviewed, acute cholecystitis is rare. In a previous study, <5% of patients with cholecystitis undergoing cholecystectomy had histologic evidence of acute cholecystitis [34]. Finally, a limited number of inflammation markers were assessed in the present study. A more comprehensive assessment of systematic inflammation and gallstone disease development merits investigation in a prospective study, and studies in other populations are needed to replicate our findings.

In conclusion, of the 13 circulating cytokines investigated, four were associated with gallstones, of which three were not associated with gallbladder cancer in a previous study, and the magnitude of the associations generally seemed to be lower than that observed for gallbladder cancer. These differences in the associations of cytokines with gallstones as opposed to gallbladder cancer suggest that the transition from normal to gallstones and from gallstones to cancer may be characterized by processes that vary in terms of the mechanism at play and/or the degree of disease-related changes. Future studies are needed to further evaluate the common pathway or mechanism associated with these cytokine signatures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1.

Characteristics of Gallstone Cases and Population-Based Controls, by Sex, Shanghai, China, 1997 – 2001

Characteristic and Category	Cases (n=109 Women, n=41 Men)		Controls (n=86 Women, n=63 Men)		$\chi 2$ *P* value [a]	
	Women (%)	Men (%)	Women (%)	Men (%)	Women	Men
Age group, years					0.217	0.08
≤ 54	26 (23.8)	4 (9.8)	12 (14.0)	13 (20.6)		
55 – 65	34 (31.2)	11 (26.8)	29 (33.7)	24 (38.1)		
≥ 66	49 (45.0)	26 (63.4)	45 (52.3)	26 (41.3)		
Education					0.018	0.207
None/Primary	46 (42.2)	6 (14.6)	45 (52.3)	15 (23.8)		
Jr. Middle	25 (22.9)	8 (19.5)	11 (12.8)	19 (30.1)		
Sr. Middle	29 (26.6)	14 (34.2)	14 (16.3)	18 (28.6)		
Some college	9 (8.3)	13 (31.7)	16 (18.6)	11 (17.5)		
Self-reported body mass index					0.178	0.866
Underweight: <18.5	8 (7.3)	2 (4.9)	11 (12.8)	5 (7.9)		
Normal: 18.5 – 24.9	65 (59.6)	30 (73.2)	56 (65.1)	46 (73.0)		
Overweight: 25 – 29.9	31 (28.4)	9 (22.0)	14 (16.3)	11 (17.5)		
Obese: ≥ 30.0	5 (4.6)	0 (0.0)	5 (5.8)	1 (1.6)		
Ever smoker					0.877	0.671
No	102 (93.6)	16 (39.0)	80 (93.0)	22 (34.9)		
Yes	7 (6.4)	25 (61.0)	6 (7.0)	41 (65.1)		
Ever drinker					0.877	0.182
No	102 (93.6)	25 (61.0)	80 (93.0)	30 (47.6)		
Yes	7 (6.4)	16 (39.0)	6 (7.0)	33 (52.4)		
Diabetes					0.091	0.278
No	93 (85.3)	35 (85.4)	80 (93.0)	58 (92.1)		
Yes	16 (14.7)	6 (14.6)	6 (7.0)	5 (7.9)		
History of cholecystitis					<0.001	<0.001
No	56 (51.4)	21 (51.2)	77 (89.5)	59 (93.7)		
Yes	53 (48.6)	20 (48.8)	9 (10.5)	4 (6.3)		

[a] Fisher's exact $\chi 2$ test was used for age group, self-reported body mass index and history of cholecystitis among men.

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Table 2.

Association between Inflammation Proteins Tested from a High Sensitivity Cytokine Panel and Gallstone risk, Shanghai, China, 1997 – 2001.

Inflammation Proteins and Category	No. of Cases (%)	No. of Controls (%)	Age- and Sex-Adjusted ORs (95% CIs) [a]	Multivariable- Adjusted ORs (95% CIs) [b]	Mutually-Adjusted ORs (95% CIs) [c]
IL-6					
Q1	19 (12.7)	38 (25.5)	reference	reference	reference
Q2	29 (19.3)	38 (25.5)	1.4 (0.7, 3.0)	1.3 (0.6, 3.2)	1.0 (0.4, 2.7)
Q3	31 (20.7)	36 (24.2)	1.7 (0.8, 3.6)	1.3 (0.5, 3.1)	0.6 (0.2, 1.8)
Q4	71 (47.3)	37 (24.8)	3.7 (1.9, 7.5)	3.2 (1.4, 7.2)	1.4 (0.5, 3.6)
P trend [d]			7.29×10^{-5}	3.26×10^{-3}	0.473
IL-10					
Q1	24 (16.0)	38 (25.5)	reference	reference	reference
Q2	24 (16.0)	37 (24.8)	1.0 (0.5, 2.1)	1.0 (0.4, 2.3)	0.5 (0.2, 1.6)
Q3	28 (18.7)	37 (24.8)	1.1 (0.5, 2.3)	1.3 (0.6, 3.2)	0.6 (0.2, 1.8)
Q4	74 (49.3)	37 (24.8)	3.4 (1.6, 6.0)	3.1 (1.4, 7.0)	1.4 (0.5, 3.8)
P trend [d]			2.42×10^{-4}	1.23×10^{-3}	0.267
IL-12 (p70)					
Q1	21 (14.0)	38 (25.5)	reference	reference	reference
Q2	14 (9.3)	37 (24.8)	0.6 (0.3, 1.5)	0.8 (0.3, 2.2)	0.9 (0.3, 2.7)
Q3	38 (25.3)	38 (25.5)	1.7 (0.8, 3.4)	1.9 (0.8, 4.5)	1.8 (0.6, 4.9)
Q4	77 (51.3)	36 (24.2)	3.6 (1.8, 7.1)	5.7 (2.5, 13.5)	5.5 (1.9, 16.2)
P trend [d]			6.01×10^{-6}	2.99×10^{-6}	0.002
IL-13					
Q1	17 (11.3)	38 (25.5)	reference	reference	reference
Q2	29 (19.3)	38 (25.5)	1.7 (0.8, 3.7)	1.7 (0.7, 4.1)	1.3 (0.5, 3.4)
Q3	49 (32.7)	36 (24.2)	3.3 (1.6, 7.0)	4.3 (1.9, 10.4)	2.8 (1.0, 7.6)
Q4	55 (36.7)	37 (24.8)	3.1 (1.5, 6.6)	3.2 (1.4, 7.5)	0.9 (0.3,2.7)
P trend [d]			7.79×10^{-4}	2.33×10^{-3}	0.890

Abbreviations: CI, confidence interval; IL: interleukin; Q, quartile; OR, odds ratio

[a] Adjusted for age and sex.

[b] Adjusted for age, sex, body mass index, education level, smoking, alcohol drinking, self-reported history of diabetes, and history of cholecystitis.

[c] Four cytokines were mutually adjusted.

[d] Two-sided *P* values for trend across protein categories were assessed with the Wald test using categorical values of the proteins with 1 degree of freedom.