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Circulating inflammatory proteins and gallbladder cancer: Potential for risk stratification to improve prioritization for cholecystectomy in high-risk regions

Jill Koshiol[a,*], Yu-Tang Gao[b], Amanda Corbel[a], Troy J. Kemp[c], Ming-Chang Shen[d], Allan Hildesheim[a], Ann W. Hsing[e,f], Asif Rashid[g], Bingsheng Wang[h], Ruth M. Pfeiffer[i], Ligia A. Pinto[c]

[a] Infections Immunoepidemiology Branch, Division of Cancer Epidemiology Genetics, National Cancer Institute, MD, USA
[b] Department of Epidemiology, Shanghai Cancer Institute, Shanghai, China
[c] HPV Immunology Laboratory, Frederick National Laboratory for Cancer Research, Leidos, Biomedical Research, Inc, Frederick, MD, USA
[d] Department of Pathology, Shanghai Cancer Center, Fudan University, Shanghai, China
[e] Stanford Cancer Institute, Stanford School of Medicine, Palo Alto, CA, USA
[f] Stanford Prevention Research Center, Department of Medicine, Stanford School of Medicine, Palo Alto, CA, USA
[g] Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
[h] Department of General Surgery, Zhongshan Hospital, School of Medicine, Fudan University, Shanghai, China
[i] Biostatitics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, MD, USA

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ABSTRACT

Background: Inflammatory proteins could help identify individuals most likely to have gallbladder cancer (GBC) among those waiting for cholecystectomy.
Methods: We analyzed 49 circulating inflammation-related proteins in 144 patients with GBC and 150 patients with gallstones. We calculated age- and sex-adjusted odds ratios (ORs) and 95% CIs for protein quantiles and GBC versus gallstones. Using proteins associated with early GBC (stage 1–2) that were selected in stepwise logistic regression, we created an inflammation score and explored the potential utility for risk stratification.
Results: 26 proteins (53%) had P values for the trend across categories ≤ 0.001, with associations for a one category increase ranging from 1.52 (95% CI: 1.20–1.94) for C−C motif ligand 4 to 4.00 (95% CI: 2.76–5.79) for interleukin (IL)-8. Soluble tumor necrosis factor receptor 2 (sTNFR2), IL-6, sTNFR1, C−C motif ligand 20 (CCL20), vascular cell adhesion molecule 1, IL-16, and granulocyte colony-stimulating factor had P values ≤ 0.001 for early GBC. Of those, IL-6, IL-16, CCL20, and STNFR1 were included in the inflammation score. In a high-risk setting with a pre-test disease risk of 10% (e.g., elderly patients) and using an inflammation score cutoff that provides 90% sensitivity, 39% of patients on the waiting list would be predicted to be positive, and 23% of those would be predicted to have GBC.
Conclusion: These results highlight the strong associations of inflammatory proteins with GBC risk and their potential clinical utility. Larger studies are needed to identify the most effective combinations of inflammatory proteins for detecting early GBC and precursor lesions.

1. Introduction

Gallstones are the main risk factor for gallbladder cancer (GBC) [1,2], a highly lethal disease with a 5-year relative survival rate of 17% across all stages [3]. Early stage (localized) tumors can be cured with cholecystectomy [4]. Thus, early detection and treatment is critical to improve survival among patients with GBC. Unfortunately, timely treatment of all individuals with gallstones through cholecystectomy is not practical given the high prevalence of gallstones, particularly in high-risk areas. In Chile, which has among the highest rates of GBC in the world, national policy dictates that individuals aged 35–49 who are diagnosed with gallstones are prioritized for cholecystectomy [5], leading to delayed treatment of older patients who are at higher GBC risk. This delay may be very important clinically since survival is much improved for early-stage cancers; 5-year survival is 41% for localized tumors, and nearly 90% for tumors confined to the muscularis, versus

* Corresponding author at: 9609 Medical Center Dr, Rm 6−E212, MSC 9767, Bethesda, MD 20892, USA.
E-mail address: koshiolj@mail.nih.gov (J. Koshiol).

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11% for regional and 3% for distant tumors [4,6].

Levels of circulating proteins related to inflammation might help stratify individuals awaiting cholecystectomy since gallbladder carcinogenesis is strongly tied to inflammation [7]. Inflammation involves multiple signaling pathways and molecules produced by various types of immune cells. Gallstones can cause inflammation [7]. Histopathologic changes indicative of inflammation in the gallbladder proceed the formation of gallstones in both animal models and humans [8–10], and gallstones have been associated with elevated levels of circulating cytokines [11]. However, circulating levels of inflammatory proteins are notably higher in patients with GBC than in patients with gallstones alone [11,12]. We previously used multiplex cytokine panels to measure these proteins in circulation and in bile and showed strong elevations in patients with GBC compared to those with gallstones [12,13].

The ability to prioritize the cholecystectomy waiting list according to the probability that GBC is present could help identify patients with early stages of GBC and thus increase the chances of curative treatment. These effects are particularly important in high-risk regions, such as Chile, Northern India, and Shanghai, China. In the present study, we evaluated the utility of using circulating inflammatory proteins to stratify patients waiting for cholecystectomy by their risk of prevalent GBC.

2. Methods

From June 1997 through May 2001, the Shanghai Biliary Tract Cancer Study enrolled 368 GBC cases [2,14]. Newly diagnosed cancer cases were identified through a rapid-reporting system established between the Shanghai Cancer Institute and 42 collaborating hospitals in 10 urban districts of Shanghai, and 774 gallstone patients were frequency matched to patients with GBC on age, sex, and hospital. Over 74% of patients with GBC were confirmed by histology. Those without pathological tissue, generally due to unresectable tumors, were evaluated by a clinical review panel of four gastrointestinal surgeons and a pathologist, who reviewed imaging data and clinical and operative reports [2]. All patients were permanent residents of urban Shanghai between the ages of 35 and 74 without a previous non-skin cancer, and provided written informed consent. The U.S. National Cancer Institute and Shanghai Cancer Institute institutional review boards approved the study.

We evaluated circulating inflammatory proteins in 144 GBC cases and 150 randomly selected gallstone patients who either had serum collected prior to surgery or did not have surgery. We used the Milliplex (EMD Millipore, Billerica, MA) and the Meso Scale Discovery (MSD) Human Vascular Injury II (Meso Scale Diagnostics LLC, Rockville, MD, USA) kits to test for 68 inflammatory proteins. For the Milliplex assay, 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. The MSD plate-based ELISA assay was performed according to the manufacturer's instructions. Briefly, serum samples were incubated with assay diluent, followed by incubation with a detection antibody. The MSD plates were then analyzed using the MSD Sector Imager 6000 plate reader and Discovery Workbench 3.0 software (Meso Scale Diagnostics LLC, Rockville, MD, USA).

Some of these proteins have been previously reported in a subset of patients in the current study, which expanded the number of patients and tested new markers [12]. Quality control evaluations led us to drop 19 of 68 proteins (see supplemental methods), leaving 49 proteins for analysis, which are listed in Fig. 1.

As performed in previous studies [15,16], cutpoints were used to create categories that were determined by the proportion of subjects with detectable values as follows: (a) for proteins detectable in ≥75% of subjects, four categories were created based on quartiles of values above the lower limit of quantitation (LLOQ) using the distribution among gallstone controls (subjects with undetectable values were included in the lowest quartile); (b) for proteins detectable in 50–75% of subjects, four categories were created where the first category included all subjects with undetectable values and the next three categories were based on tertiles of values above the LLOQ; (c) for proteins detectable in 25–50% of subjects, three categories were created where the first category included all subjects with undetectable values and the next two categories were based on a median split of the values above the LLOQ; (d) for proteins detectable in < 25% of subjects, two categories were created: one for undetectable values and the other for values above the LLOQ. These proteins were modeled both categorically and ordinally (coded as 1, 2, 3, 4) to evaluate linear trend. For simplicity, we provide the ordinal ORs, which reflect the linear change per category.

Univariate associations between sociodemographic and behavioral characteristics and case-control status were investigated using the Kruskal-Wallis test for difference in medians and chi-square tests for categorical comparisons. To best control for potential differences by assay lot, we fit logistic models conditional on lot to calculate ORs and 95% CIs for associations between inflammatory proteins and GBC versus gallstones.

All models were adjusted for age (≤54, 55–65, ≥66) and sex (male/female). In addition, we conducted stepwise linear regression among population-based controls to determine whether inflammation marker levels above the LLOQ were associated with: education (none/primary, junior middle, senior middle, some college), ever drinking, ever smoking, categorical body mass index (underweight, normal weight, overweight, obese), fasting status (fasting/not fasting), and history of diabetes. We required $p < 0.05$ for a variable to be entered into a model and $p < 0.01$ for that variable to be retained in the model. After identifying potential confounders through stepwise linear regression, we fit logistic regression models to determine whether the covariate changed the OR for the association between the categorical inflammation protein and GBC by more than 10% while adjusting for age and gender. No covariate changed the OR by more than 10%, so, the final models included only age and sex. At the time of analysis, we identified 11 samples (4 from patients with GBC and 7 from patients with gallstones) that were collected after surgery, but exclusion of these samples did not substantively affect the results [12]. We also considered multiple comparisons by applying a Bonferroni correction of $\alpha = 0.001$ (0.05/49 proteins analyzed).

After examining associations for all patients with GBC, we fit models restricted to early/localized GBC (stage 1 and 2) compared to all controls. For proteins that were associated with early GBC, we conducted stepwise logistic regression using $\alpha = 0.05$ to determine which proteins were associated with GBC (all cases combined) compared to gallstones taking the other proteins into account. For those proteins that remained in the model, we created an inflammation score. We created this score by summed the categorical values (i.e., 1, 2, 3, or 4) for the level of each protein weighted by the log-OR from the model for that protein and gallbladder cancer risk (using all gallbladder cancer cases, regardless of stage). We then divided the score into four categories based on the distribution in the gallstone controls.

Finally, we explored the potential clinical utility of proteins and the summary inflammation score to identify early GBC. We set the sensitivity for each at 90%, 80%, or 70% and varied the prevalence of GBC (i.e., assumed risk before testing for the marker) from 1% to 10%. We then used the biomarker webtool available at https://analysistools.nci.nih.gov/biomarkerTools/, originally described by Wentzensen and Wacholder [17], to estimate the specificity, number positive per 1000 cholecystectomy patients screened, and positive predictive value (PPV, the risk of disease after a positive test).

3. Results

GBC patients were slightly older than gallstone patients (median age 67 versus 65, $P = 0.005$) and were also less educated ($P = 0.0002$), but