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# The Correlation Between Serum Chemokines and Clinical Outcome in Patients with Advanced Biliary Tract Cancer

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## Abstract

*BACKGROUND:* Biliary tract cancers (BTCs) are known to have a dismal prognosis. A number of chemokines play important roles in the progress of BTCs. However, the serum levels of chemokines in BTCs have not yet been explored. *METHODS:* The sera of healthy donors (n = 8) and patients with BTCs who were enrolled in second line sunitinib trials (n = 27) were collected. The concentrations of three kinds of chemokines (CXCL5, CXCL8 and CXCL12) were measured using ELISA assay. The median concentrations of chemokines were compared between healthy donors and BTC patients and the role of chemokines as a prognostic biomarker was examined. *RESULTS:* BTC patients generally had higher serum levels of CXCL5 and CXCL12 compared to healthy donors. Patients with cholangiocarcinoma showed significantly higher levels of serum CXCL12 than patients with gallbladder cancer. In survival analysis, only CXCL12 level showed a prognostic impact on overall survival (median OS: 6.9 vs. 0.9 months in low CXCL12 vs. high CXCL12, respectively; $P = .008$). High CXCL5 levels were also correlated with poor survival without statistical insignificance (median OS: 6.2 vs. 2.0 months in low CXCL5 vs. high CXCL5, respectively; $P = .070$). *CONCLUSIONS:* There was a significant difference in OS according to the level of CXCL12, suggesting that serum CXCL12 levels may be a useful surrogate marker for clinical outcome in advanced BTCs.

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## Introduction

Biliary tract cancers (BTCs), including cholangiocarcinoma and gallbladder cancer, are low-incidence cancers [1], but relatively more common in Asia and Latin America [2]. Most patients (>65%) are diagnosed with unresectable disease and there is a high relapse rate in the minority of patients who undergo potentially curative surgery [3]. Combination chemotherapy with gemcitabine and platinum agents seems to be a reasonable treatment option as first-line treatment based on randomized phase III trial (ABC-02) [4]. However, prognosis of advanced and metastatic BTCs is poor with a five-year survival rate of about 2% for stage IV BTCs [5]. Therefore, it is urgent to uncover the molecular mechanisms of BTCs and identify potential therapeutic targets to improve prognosis.

Chemokines, small molecular weight proteins (approximately 8-13 kDa), are chemotactic cytokines specialized in regulating the migration of immune cells into damaged or diseased organs in response to pro-inflammatory stimuli [6]. Together with their corresponding receptors, chemokines promote the extravasation of immune cells from the circulation into injured tissue and regulate the migration of immune cells through the tissue. To date, about 50 different chemokines and 20 difference chemokine receptors have been identified [7,8]. Over the past few years, studies have increasingly shown that chemokines play an important role in several aspects of tumor progression [9–12]. Chemokines released by tumor and stromal cells can induce the expression and distribution of tumor-associated leukocytes, trigger angiogenesis and generate fiber keratinocytes [13,14]. Chemokines released into the matrix can also directly contribute to the growth of malignant cells [9,14]. C-X-C chemokine receptor 4 (CXCR4) is frequently overexpressed in cancer

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**Table 1.** Baseline Characteristics of Patients (N = 27)

| Variables | No. of Patients (N = 27) | % of Patients |
|---|---|---|
| Age, median (range), years | 55 (38-75) | |
| ≤65 | 23 | 85.2 |
| >65 | 4 | 14.8 |
| Sex | | |
| Male | 17 | 63.0 |
| Female | 10 | 37.0 |
| Primary site | | |
| Intrahepatic duct | 12 | 44.4 |
| Extrahepatic duct | 4 | 14.8 |
| Gallbladder | 11 | 40.7 |
| Disease status | | |
| Recurrent | 4 | 14.8 |
| Primarily metastatic | 23 | 85.2 |
| First-line chemotherapy | | |
| Gemcitabine/platinum combination | 19 | 70.4 |
| 5FU/platinum combination | 8 | 29.6 |
| Site of metastasis | | |
| Liver | 22 | 81.5 |
| Lymph node | 22 | 81.5 |
| Lung | 9 | 33.3 |
| Peritoneum | 8 | 29.6 |
| Pleural | 3 | 11.1 |
| Bone | 2 | 7.4 |
| CA 19-9 | | |
| ≤37 IU/mL | 9 | 33.3 |
| >37 IU/mL | 18 | 66.7 |

cells, and chemokine ligand 12 (CXCL12)-CXCR4 interactions underlie invasiveness in a variety of cancers [11,12].

There are a few reports about the effects of chemokines and chemokine reception interaction in BTCs, such as CXCL12-CXCR4 or chemokine ligand 5 (CXCL5)-C-X-C chemokine receptor 2 (CXCR2) [15–17]. However, these have all been examined in tumor tissues. Therefore, we explored whether circulating chemokines are detectable in enzyme-linked immunosorbent assay (ELISA) and if they have prognostic impact on survival in patients with BTCs.

## Materials and Methods

### *Patients*

From May 2009 to October 2010, a total of 56 patients were enrolled in sunitinib BTC trials and blood samples from 27 patients were collected for biomarker analysis. The eligibility criteria and design of this study were previously described [18]. Informed consent was signed and obtained from all patients before being involved in the study. The Ethics Committee of Samsung Medical Center approved and supervised this study.

### *Blood Samples and ELISA Assay*

Blood samples (5ml) were drawn from BTC patients or healthy donors before chemotherapy or after surgery, respectively. After collection, the samples were kept at room temperature for 2 hours to allow clotting and then were immediately centrifuged at 2200 rpm for 15 minutes at 4°C and were the cryopreserved at −80°C until ELISA assays were run. The level of serum chemokine was quantified using a commercially available ELISA kit (R&D) according to the manufacturer's instructions.

*Selection of Cut-Off Value for Chemokines.* Since there was no reference range available for serum chemokine levels, the "minimum P value" approach [19–21] was applied to estimate an optimal cut-off of chemokines for the best separation of patients' PFS/OS by X-tile software [22], version 3.6.1 (Yale University, New Haven, CT). X-tile plots can be used to divide a population into two levels (low and high level) and provide an "on-the-fly" histogram with an associated Kaplan-Meier curve, and the best P value is available after rigorous statistical evaluation by X-tile.

### *Statistical Analyses*

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). The independent *t*-test or Mann-Whitney *U*-test were used for testing statistical significance of mean differences between the two groups. The progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan-Meier estimates method. The log-rank test was applied to compare survival between the two groups. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. A *P*-value of less than 0.05 was regarded as statistically significant.

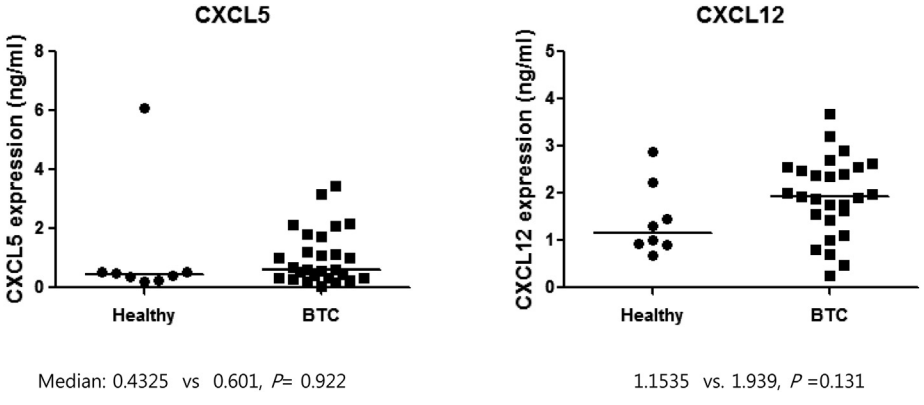
## Results

### *Patients' Characteristics*

This analysis included 27 patients who received sunitinib as second-line treatment for advanced BTCs. Baseline characteristics are presented in Table 1. The median age was 55 years (range, 38-75 years) and patients were predominantly male (63.0%). Twelve patients (44.4%) had intrahepatic duct cancer, 4 (14.8%) had extrahepatic duct cancer and 11 (40.7%) had gallbladder cancer. Two-thirds of patients (66.7%) had high CA19-9 level at baseline.

### *Chemokine Level*

The median serum CXCL5 levels were 0.4325 ng/mL and 0.601ng/mL in healthy donors and patients with BTCs, respectively.




Median: 0.4325 vs 0.601, *P*= 0.922

1.1535 vs. 1.939, *P* =0.131

**Figure 1.** Distribution of serum chemokine level in healthy donors and patients with BTC

**Table 2.** Median Serum Chemokine Levels According to Clinical Features

| Variables | No. of Patients (%) | Median CXCL5 (ng/mL) | *P* | Median CXCL12 (ng/mL) | *P* |
|---|---|---|---|---|---|
| Age, years | | | .617 | | .020 |
| ≤65 | 23 (85.2) | 0.681 | | 1.996 | |
| >65 | 4 (14.8) | 0.460 | | 1.275 | |
| Sex | | | .488 | | .233 |
| Male | 17 (63.0) | 0.579 | | 1.939 | |
| Female | 10 (37.0) | 0.681 | | 1.938 | |
| Primary site | | | .531 | | .001 |
| Intrahepatic/extrahepatic | 16 (59.3) | 0.590 | | 2.390 | |
| Gallbladder | 11 (40.7) | 0.681 | | 1.115 | |
| First-line chemotherapy | | | .943 | | .402 |
| Gemcitabine-based | 19 | 0.579 | | 1.996 | |
| 5FU_based | 8 | 1.055 | | 1.847 | |
| Liver metastasis | | | .683 | | .452 |
| Yes | 22 (81.5) | 0.601 | | 1.746 | |
| No | 5 (18.5) | 0.641 | | 1.967 | |
| CA 19-9 | | | .900 | | .372 |
| ≤37 IU/mL | 9 (33.3) | 0.681 | | 1.996 | |
| >37 IU/mL | 18 (66.7) | 0.590 | | 1.811 | |

As for CXCL12, the median serum levels were 0.1535 ng/mL and 1.939ng/mL in healthy donors and patients with BTCs, respectively (Figure 1). BTC patients generally had higher serum chemokine levels compared to healthy donors, but this difference was not statistically significant ($P$ = .922 and $P$ = .131 in CXCL5 and CXCL12, respectively). Serum CXCL8 level was very low in both healthy donors and BTC patients.

### *Chemokine Level According to Clinical Features*

Age, sex, primary site, first-line chemotherapy regimen, presence of liver metastasis, and CA19-9 level did not influence the serum CXCL5 or CXCL12 levels with the exception of higher CXCL12 level in intrahepatic or extrahepatic duct cancer (Table 2). The median serum CXCL12 level was 2.390 ng/mL from patients with intrahepatic or extrahepatic duct cancer, and 1.115 ng/mL from patients with gallbladder cancer. This difference was statistically significant ($P$ = .001).

### *Survival Analysis*

The results of univariate analysis for overall survival are shown in Table 3. Age, sex, primary site, disease status, previous chemotherapy regimen, liver metastasis, and CA19-9 level failed to show any relation to survival.

According to the "minimum $P$ value" approach, X-tile software was applied to estimate the optimal cut-off of chemokines for the best separation of patients' OS. Here, $P$ = .014 was the minimum $P$ value when CXCL5 arrived at 2.081, while $P$ = .016 for CXCL12 at 2.630. Univariate analysis with these cut-off points showed that high CXCL12 level was correlated with poor overall survival (median OS, 6.9 vs. 0.9 months in low vs. high CXCL12 level groups; $P$ = .008). High CXCL5 levels also tended to be associated with shorter survival, but the difference was not statistically significant (median OS, 6.2 vs. 2.0 months in low vs. high CXCL5 level groups; $P$ = .070) (Figure 2). In multivariate analysis for overall survival, only CXCL12 level was a statistically significant prognostic factor with hazard ratio of 4.609 (95% CI, 1.144-18.560; $P$ = .032). (See Table 4).

**Table 3.** Prognostic Factors for Overall Survival in Univariate Analysis

| Variables | No. of Patients (N = 27) | Median OS (Months) | 95% CI | *P* |
|---|---|---|---|---|
| Age, years | | | | .615 |
| ≤65 | 23 | 6.1 | 3.454-8.746 | |
| >65 | 4 | 11.5 | 0.000-24.374 | |
| Sex | | | | .7.32 |
| Male | 17 | 6.1 | 2.518-9.682 | |
| Female | 10 | 3.4 | 0.000-7.894 | |
| Primary site | | | | .741 |
| Intrahepatic/extrahepatic | 16 | 6.1 | 3.804-8.396 | |
| Gallbladder | 11 | 11.5 | 4.620-18.380 | |
| Disease status | | | | .686 |
| Recurrent | 4 | 6.2 | 0.000-14.284 | |
| Primarily metastatic | 23 | 6.1 | 2.254-9.946 | |
| First-line chemotherapy | | | | .329 |
| Gemcitabine-based | 19 | 6.4 | 3.017-9.183 | |
| 5FU_based | 8 | 4.2 | 0.8-13.822 | |
| Liver metastasis | | | | .427 |
| Yes | 22 | 6.1 | 3.657-8.543 | |
| No | 5 | 11.5 | 0.000-29.965 | |
| CA 19-9 | | | | .874 |
| ≤37 IU/mL | 9 | 4.2 | 2.004-6.396 | |
| >37 IU/mL | 18 | 6.2 | 3.935-8.465 | |
| CXCL5 | | | | .070 |
| ≤2.081 ng/mL | 23 | 6.2 | 3.748-83652 | |
| >2.081 ng/mL | 4 | 2.0 | 0.000-4.025 | |
| CXCL12 | | | | .008 |
| ≤2.630 ng/mL | 23 | 3.9 | 5.888-7.912 | |
| >2.630 ng/mL | 4 | 0.9 | 0.000-4.232 | |

## Discussion

Compared to other malignancies, BTCs are generally characterized by aggressive behavior, such as strong proliferation, invasion and early metastasis. Many factors such as adhesion molecules, proteases, cytokines and chemokines are involved in these processes [23]. With respect to the CXCR4-CXCL12 axis, Ohira et al. [24] demonstrated that CXCR4 was mainly expressed in intrahepatic cholangiocarcinoma cells and CXCL12 in stromal fibroblasts, and the interaction of CXCL12 released from fibroblasts and CXCL4 expressed on tumor cells may be actively involved in intrahepatic cholangiocarcinoma migration, suggesting CXCR4 could be a therapeutic target to prevent cancer invasion. This possibility was confirmed by Gentilini et al. [15] using AMD3100, an antagonist of CXCR4, and Tan et al. [25] using siRNA targeting CXCR4. Furthermore, the canonical Wnt pathway was suggested as an underlying mechanism of CXCL12/CXCR4 signaling on cholangiocarcinoma progression [23]. In addition, Lee et al. showed that the expression of CXCL12 is significantly associated with a high histologic grade and nodal metastasis, and that CXCL12 expression is an independent risk factor for patient survival in GB cancer [26]. As for the CXCL5-CXCR2 axis, CXCL5 was a poor prognostic factor for survival in patients who had resection for cholangiocarcinoma [17]. CXCR2 was up-regulated in cholangiocarcinoma compared to adjacent liver tissue and had prognostic value in patients with cholangiocarcinoma [27]. All of these studies have been conducted in tumor specimens of BTCs and there is no data about serum chemokines in BTCs. In other types of cancers, a few studies evaluating serum chemokines have been

**Table 4.** Multivariate Analysis for Overall Survival

| Variables | Hazard Ratio | 95% CI | *P* |
|---|---|---|---|
| Age, >65/≤65 | 0.644 | 0.126-3.302 | .598 |
| Primary site, GB cancer/duct cancer | 1.094 | 0.536-2.233 | .806 |
| CA19-9, high/low | 1.019 | 0.295-3.522 | .976 |
| CXCL5, high/low | 3.840 | 0.620-23.806 | .148 |
| CXCL12, high/low | 4.609 | 1.144-18.560 | .032 |

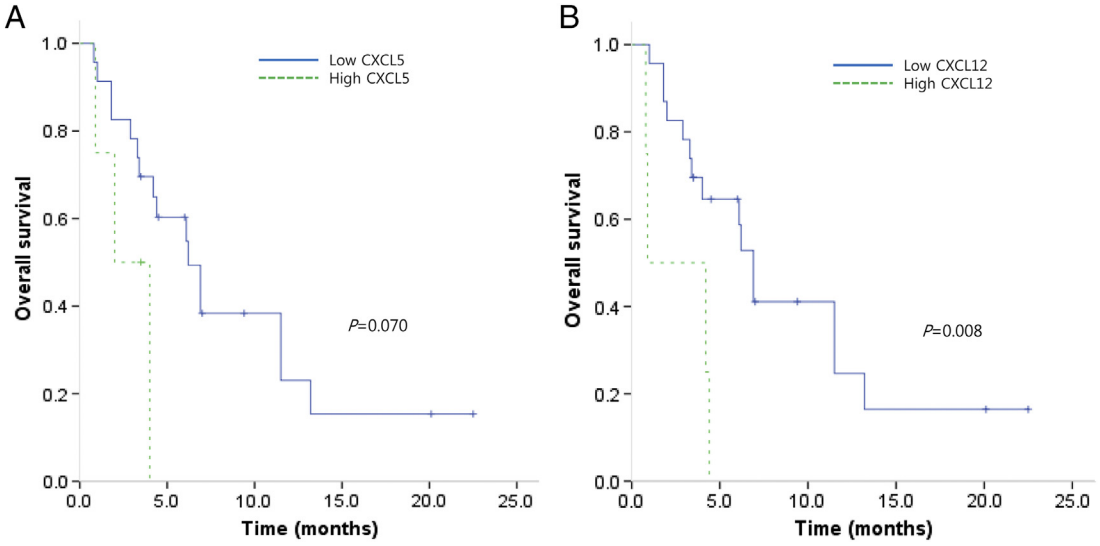



**Figure 2.** (A) Kaplan-Meier curve of overall survival (OS) by CXCL5 levels ($P$ = .070). (B) Kaplan-Meier curve of OS by CXCL12 levels ($P$ = .008).

reported, such as CXCL12 in colon cancer, CCL22 in breast cancer or CCL5 in gastric cancer [28–30].

As the initial report of serum chemokines in BTCs, our study showed that CXCL5 and CXCL12, which have been reported as prognostic factors in tumor tissues of BTCs patients, can be detected in serum by ELISA method and BTC patients generally have higher levels of chemokines compared to healthy donors. In addition, serum CXCL5 and CXCL12 levels were associated with patient prognosis although the p-value was not significant for CXCL5. Even in multivariate analysis, serum CXCL12 remained a meaningful factor for survival.

The CXCL12/CXCR4 axis is an attractive target for various cancer types and several compounds belonging to different chemical classes are able to interact with the active site of CXCR4. Targeted therapies using CXCR4 antagonists represent a promising approach for the treatment of cancer [31]. Currently, plerixafor, a CXCR4 antagonist, is used as an immunostimulant to mobilize hematopoietic stem cell in hematologic malignancies and is being tested in phase I trial with pancreatic, ovarian, colorectal cancers (NCT02179970). A clinical trial with another CXCR4 antagonist, LY2510924 in combination with immune checkpoint inhibitor durvalumab, is now ongoing in solid tumors (NCT02737072). Further evaluation is needed to verify the role of serum CXCR12 level as a predictive marker for CXCR4 antagonists and efficacy of CXCR4 antagonists in BTCs.

This study had several limitations. First, this study included only 8 healthy donors and 27 BTC patients and the subgroups were too small. Small sample size and selection bias of the current study may make definitive conclusions difficult. Second, as there was no reference range for CXCL5 and CXCL12, we determined the optimal cutoff using the X-tile program. It was an arbitrary cutoff, and therefore validation in a large sample size is needed. Although the significance of these studies is inconclusive due to small sample sizes, these intriguing observations show that serum CXCL12 levels may be a useful surrogate marker of clinical outcome in advanced BTCs. These data may provide useful information and background for future research into chemokines in BTCs.

## Declaration of Interest

The authors have no competing interests to declare.

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