

Original Article

Serum total bile acid correlates with macrophage-related immune features in colorectal cancer patients after cholecystectomy

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Abstract: Cholecystectomy has been associated with an elevated risk of colorectal cancer (CRC), potentially mediated by alterations in bile acid metabolism. This single-center retrospective case-control study aimed to investigate the clinical significance of serum total bile acid (TBA) levels in patients with CRC after cholecystectomy, their association with farnesoid X receptor (FXR) expression, and their relationship with tumour-associated macrophage (TAM)-related immune features within the tumour microenvironment. A total of 216 post-cholecystectomy patients with CRC and 209 post-cholecystectomy controls without CRC were enrolled in the study. Serum TBA levels were quantified and statistically compared between the two groups. Immunohistochemical analysis was performed on tumour tissues to assess FXR expression and TAM markers (CD163, CD68). Pearson correlation analysis was performed to examine the associations among serum TBA levels, FXR expression, and TAM-related immune features. Serum TBA levels were significantly higher in the CRC group ($8.74 \pm 4.45 \mu\text{mol/L}$) compared to the Control group ($4.61 \pm 2.90 \mu\text{mol/L}$). ROC analysis showed an AUC of 0.79 (95% CI: 0.74-0.83). Elevated TBA levels were associated with advanced disease features, including higher TNM stage and lymph node metastasis. Immunohistochemistry revealed increased FXR expression and a higher proportion of CD163⁺ TAMs in the high TBA group, while the proportion of CD68⁺ TAMs was reduced. Pearson correlation analysis showed that TBA levels were positively correlated with FXR⁺ cells ($r=0.57$, $P < 0.001$) and CD163⁺ macrophages ($r=0.51$, $P < 0.001$), and negatively correlated with CD68⁺ macrophages ($r=-0.44$, $P < 0.001$). Elevated serum TBA levels were associated with increased FXR expression and altered TAM characteristics in CRC after cholecystectomy. These findings indicate that TBA levels may represent a biomarker associated with CRC-related clinicopathological and immune characteristics.

Keywords: Colorectal cancer, cholecystectomy, total bile acid, farnesoid X receptor, tumor-associated macrophages

Introduction

Cholecystectomy is the standard surgical treatment for benign gallbladder disease and well established as a safe procedure. Since Hoare first suggested a possible association between cholecystectomy and colorectal cancer (CRC) in 1974 [1], the long-term oncological effects of cholecystectomy, particularly its possible association with gastrointestinal malignancies, have received increasing attention [2, 3]. Despite extensive case-control, cohort, and meta-

analytic studies, the link between cholecystectomy and CRC risk remains inconclusive [4]. Although the systematic review by Coats and Shimi reported no significant association, growing evidence from Western cohorts suggests an increased risk of colorectal cancer-particularly right-sided colon cancer, after cholecystectomy, with a higher risk reported in female patients [5-8]. These discrepancies may result from differences in study design, populations, and control of confounding factors. Nonetheless, growing evidence supports a biologically

plausible connection, suggesting altered bile acid metabolism as a potential contributor [9].

Altered bile acid metabolism after cholecystectomy has been proposed as a potential contributor to colorectal carcinogenesis. Changes in bile storage and release may modify intestinal exposure to secondary bile acids, particularly deoxycholic acid (DCA). DCA and other secondary bile acids have been associated with oxidative stress, DNA damage, and abnormal epithelial proliferation [10, 11]. Beyond their detergent function, bile acids also act as signalling molecules through receptors such as the farnesoid X receptor (FXR). FXR is involved in the regulation of bile acid synthesis, epithelial barrier function, inflammation, and cell turnover [12, 13]. Experimental studies have linked FXR deficiency to increased colonic tumour burden [14], while reduced FXR expression has been reported in colorectal cancer tissues [15].

Accumulating evidence suggests that bile acid metabolism and FXR signaling are associated with changes in the tumour immune microenvironment [16, 17]. However, the direct relationships between serum TBA levels, FXR expression, and TAM-related immune features in post-cholecystectomy colorectal cancer remain insufficiently explored [18].

To address this gap, this study examines the clinical and immunological features of CRC following cholecystectomy. Specifically, we aim to: (1) evaluate serum TBA levels and their association with CRC status; (2) examine the association between serum TBA levels and FXR expression; and (3) investigate the association between FXR expression and TAM-related markers in the tumor microenvironment. Therefore, this study aimed to clarify the associations among serum TBA levels, FXR expression, and TAM-related immune features in post-cholecystectomy CRC.

Materials and methods

Study population

This was a single-center retrospective case-control study conducted at the People's Hospital of Xinjiang Uygur Autonomous Region. Participants between January 2019 and December 2024 were analyzed. All participants had a confirmed history of cholecystectomy for

benign gallbladder disease, verified by surgical records or preoperative abdominal imaging. The CRC group included patients who underwent surgical resection at our institution and had a histopathologically confirmed diagnosis of CRC. The control group included individuals who underwent health examinations at our institution and had no evidence of CRC or other colorectal neoplasia. Controls were frequency-matched to CRC patients by time since cholecystectomy. The study was approved by the ethics committee of the People's Hospital of Xinjiang Uygur Autonomous Region (#KY20260-12923, approved date: Jan 29, 2026). Informed written consent was obtained from the participants.

Inclusion criteria

For the cholecystectomy with CRC group, eligible participants were aged ≥ 18 years, irrespective of sex, with a histopathologically confirmed diagnosis of colorectal cancer following surgical resection. Individuals were required to have no history of other major abdominal surgery except cholecystectomy, and no history of neoadjuvant chemotherapy, radiotherapy, or immunotherapy before surgery.

The control group after cholecystectomy included individuals aged ≥ 18 years with complete clinical data, a history of cholecystectomy and no history of malignancy, significant metabolic dysfunction, or inflammatory bowel disease. Colorectal neoplasia was excluded by colonoscopy performed within 6 months before enrollment.

Controls were frequency-matched to the CRC group according to the duration after cholecystectomy.

Exclusion criteria

Participants in both groups were excluded if they had received chemotherapy, radiotherapy, or immunotherapy before enrollment, or had a documented history of other benign or malignant neoplasms (except the primary CRC in the CRC group), inflammatory bowel disease, or chronic liver disease, including viral hepatitis, alcoholic hepatitis, non-alcoholic fatty liver disease, or drug-induced hepatitis.

Participants were also excluded if they had other major abdominal surgery except cholecystectomy, incomplete clinical data, or unavailable serum samples.

Sample size estimation

The sample size was estimated based on preliminary serum TBA measurements using a two-independent-sample comparison approach. Assuming mean serum TBA levels of 8.3 $\mu\text{mol/L}$ in CRC patients and 3.8 $\mu\text{mol/L}$ in controls, with a pooled standard deviation of approximately 4.0 $\mu\text{mol/L}$, a two-sided α of 0.05 and 80% power yielded a minimum sample size of 182 participants per group. The final cohort included 216 CRC patients and 209 controls.

Indications for cholecystectomy

Indications for cholecystectomy included gallstones with chronic cholecystitis, gallbladder polyps, and other benign gallbladder diseases. These indications comprised gallstones with chronic cholecystitis (82.7%), gallbladder polyps (11.3%), and other benign gallbladder diseases (6.0%). Their distribution did not differ significantly between the two groups ($P=0.584$).

Cholecystectomy history and indications were verified from operative records or preoperative imaging, including computed tomography, magnetic resonance imaging, and abdominal ultrasonography.

Serum sample collection and biochemical analysis

Venous blood samples were collected in serum-separating tubes after an overnight fast of at least 8 hours. For CRC patients, blood samples were collected within 1 week before radical colorectal cancer surgery; for controls, blood samples were collected on the day of the health examination. Serum total bile acid (TBA) levels were measured using a microplate-based assay kit (E003-2-1; Nanjing Jiancheng, China), according to the manufacturer's protocol.

Tissue processing and immunohistochemistry

Surgical tissue specimens were fixed in 4% paraformaldehyde for 24-48 hours, paraffin-embedded, and cut into 4 μm -thick sections for immunohistochemical analysis. Antigen retrieval

was performed using citrate buffer (pH 6.0) by high-pressure heating for 15 minutes, followed by cooling to room temperature. After blocking endogenous peroxidase activity, sections were incubated with primary antibodies at 4°C overnight. Primary antibodies included anti-CD68 (ab955), anti-CD163 (1:200; ab189915), and anti-FXR (NR1H4, ab228949), all purchased from Abcam (Cambridge, UK). Immunostaining was performed using a streptavidin-peroxidase immunohistochemistry kit, followed by 3,3'-diaminobenzidine visualization and haematoxylin counterstaining. Human tonsil tissue was used as a positive control for CD68 and CD163 staining, while normal liver tissue was used as a positive control for FXR staining. Negative controls were prepared by replacing the primary antibodies with phosphate-buffered saline.

Immunohistochemical staining was independently evaluated by two experienced pathologists who were blinded to the clinical data. For each section, five randomly selected non-overlapping high-power fields ($\times 400$) were examined. For CD163 assessment, only stromal macrophages showing membranous or cytoplasmic staining were counted as positive cells, whereas nonspecific staining in tumor epithelial cells was excluded. Positive and total cells were counted in each field, and the proportion of positive cells was calculated. The mean proportion across the five fields was used as the final score for each sample. The inter-observer agreement was assessed using the intraclass correlation coefficient (ICC), which was 0.89 (95% CI: 0.85-0.92).

Statistical analysis

The distribution of continuous variables was assessed using the Kolmogorov-Smirnov test. Normally distributed continuous variables were presented as mean $\pm$ standard deviation (SD), whereas categorical variables were presented as n (percentage). Continuous variables were compared using the Mann-Whitney U test for non-normally distributed data or an unpaired t-test with Welch's correction for normally distributed data with unequal variances. Categorical variables were compared using Fisher's exact test. Pearson correlation analysis was used for variables meeting the assumptions of parametric testing. Receiver operating characteristic (ROC) curve analysis was performed to

assess the discriminatory ability of serum TBA levels. The area under the curve (AUC) with 95% confidence intervals was calculated, and the optimal cutoff was determined using the maximum Youden index. Internal validation was performed using 1,000 bootstrap resamples. All statistical analyses were performed using SPSS version 26.0 (IBM, Armonk, NY, USA) and GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). A two-sided *p* value < 0.05 was considered statistically significant.

Multivariable logistic regression analyses were performed to evaluate the associations of serum TBA levels and CRC status, advanced TNM stage, and lymph node metastasis. Covariates were selected based on clinical relevance and included demographic characteristics, metabolic factors, and cholecystectomy-related variables. Multivariable linear regression was used to assess the associations between serum TBA levels and tissue marker expression. Adjusted effect estimates with 95% confidence intervals were reported. Variance inflation factors (VIFs) were calculated to assess multicollinearity. Missing data were handled using complete-case analysis.

Results

Clinical characterization of colorectal cancer following cholecystectomy

A total of 250 potentially eligible participants were screened for each group (Figure S1). In the CRC group, 34 participants were excluded, including those with other malignancies, chronic liver disease, inflammatory bowel disease, prior neoadjuvant chemoradiotherapy, or incomplete clinical data, leaving 216 patients for the final analysis. In the control group, 41 participants were excluded because of colorectal neoplasia detected on colonoscopy, other malignancies, chronic liver disease, inflammatory bowel disease, or incomplete clinical data, leaving 209 controls for the final analysis. A total of 216 patients with CRC and 209 non-CRC (Control) individuals were enrolled in the study. The control group was frequency-matched to the CRC group by time since cholecystectomy. The two groups were matched for the duration since cholecystectomy (CRC: 9.14 ± 4.05 years; Control: 8.62 ± 3.66 years; *P*=0.397). Baseline demographic and clinical characteristics, including age, sex, place of resi-

dence, presence of diabetes mellitus or hypertension, and family history of CRC, were comparable between groups (Table 1). No statistically significant differences were observed in age distribution (*P*=0.272), sex (*P*=0.628), residence (*P*=0.332), prevalence of diabetes mellitus (*P*=0.669), hypertension (*P*=0.422), or family history of CRC (*P*=0.148) (Table 1).

Serum total bile acid levels and their discriminatory performance in post-cholecystectomy colorectal cancer

To assess the discriminatory ability of serum TBA levels between CRC patients and controls following cholecystectomy, serum TBA concentrations were compared between the groups. Serum TBA levels were significantly higher in the CRC group, with a mean concentration of 8.74 ± 4.45 $\mu\text{mol/L}$ (95% CI: 8.14-9.34), compared with 4.61 ± 2.90 $\mu\text{mol/L}$ (95% CI: 4.21-5.00) in the Control group (*P* < 0.001, Figure 1A). These findings indicate an association between higher serum TBA levels and CRC status after cholecystectomy. Receiver operating characteristic (ROC) analysis showed that serum TBA levels discriminated between CRC patients and controls, with an area under the curve (AUC) of 0.79 (95% CI: 0.74-0.83, *P* < 0.001; Figure 1B). The optimal cutoff value of 5.45 $\mu\text{mol/L}$ was determined using the maximum Youden index, corresponding to a sensitivity of 75.46% and a specificity of 71.77%. Internal validation using 1,000 bootstrap resamples yielded a corrected AUC of 0.78. These results support the discriminatory performance of serum TBA levels for CRC status in this post-cholecystectomy cohort.

Association between serum TBA levels and clinicopathological characteristics in CRC patients after cholecystectomy

Among 216 CRC patients who underwent cholecystectomy, individuals were divided into high and low serum TBA groups based on the median TBA concentration. No significant differences were observed between the groups in age, sex, tumour location, or serum tumor markers including CA125, CA19-9, and CEA (all *P* > 0.05). In contrast, higher serum TBA levels were significantly associated with TNM stage and lymph node metastasis. Specifically, the high-TBA group had a greater proportion of

Table 1. Demographic and clinical characteristics of patients with CRC and without colorectal cancer (Control) after cholecystectomy

Characteristics	Control (n=209)	CRC (n=216)	p-value
Age (years)			
< 50	61 (29.2%)	52 (24.1%)	0.272
≥ 50	148 (70.8%)	164 (75.9%)	
Gender			
Male	110 (52.6%)	119 (55.1%)	0.628
Female	99 (47.4%)	97 (44.9%)	
Residence			
City	96 (45.9%)	110 (50.9%)	0.332
Country	113 (54.1%)	106 (49.1%)	
Diabetes mellitus			
Yes	58 (27.8%)	65 (30.1%)	0.669
No	151 (72.2%)	151 (69.9%)	
Hypertension			
Yes	73 (34.9%)	84 (38.9%)	0.422
No	136 (65.1%)	132 (61.1%)	
Family history of CRC			
Yes	12 (5.7%)	21 (9.7%)	0.148
No	197 (94.3%)	195 (90.3%)	
Duration after cholecystectomy (years)	8.62 ± 3.66	9.14 ± 4.05	0.397

The data are shown as mean ± standard deviation or n (percentage). The comparisons of data were done by the Mann-Whitney test (for duration after cholecystectomy) or Fisher's exact test (for binary variables). CRC, colorectal cancer; SD, standard deviation.

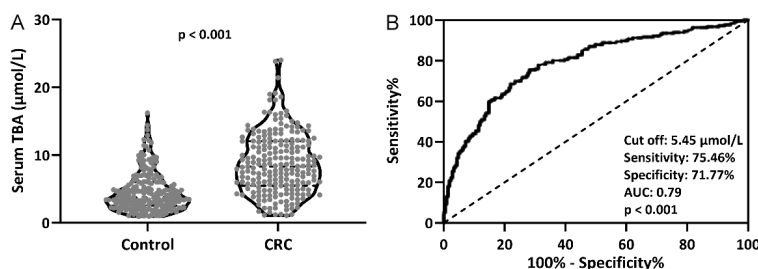


Figure 1. Serum total bile acid (TBA) concentrations and their exploratory discriminative performance for colorectal cancer in patients after cholecystectomy. A. Comparison of serum TBA levels between patients with colorectal cancer (CRC, n=216) and without colorectal cancer (Control, n=209) after cholecystectomy. The p-value was calculated using the Mann-Whitney test. B. Receiver operating characteristic (ROC) analysis of serum TBA level for the diagnosis of CRC after cholecystectomy. The area under the curve (AUC) with its 95% confidence interval, the optimal cut-off determined by the Youden index (5.45 μmol/L), and the corresponding sensitivity and specificity are indicated.

patients with advanced TNM stage (stage III-IV: 60.2% vs. 38.9%, $P=0.003$) and lymph node metastasis (57.4% vs. 38.0%, $P=0.006$) (Table 2). Regarding pathological grade, poorly differentiated tumours were more frequent in the low-TBA group than in the high-TBA group

(66.7% vs. 51.9%, $P=0.038$), whereas moderately to well-differentiated tumours showed the opposite pattern (33.3% vs. 48.1%). Given the multiple comparisons, this finding was considered exploratory. Overall, serum TBA levels were associated with selected clinico-pathological characteristics in CRC patients after cholecystectomy.

Multivariable analysis of serum TBA levels and CRC-related outcomes

To further evaluate whether serum TBA levels were associated with CRC status and tumour-related characteristics, multivariable regression analyses were performed after adjustment for potential confounding factors. For the comparison between CRC patients and non-CRC controls, multivariable logistic regression analysis was performed with CRC status

Table 2. Serum TBA level and clinicopathological factors in colorectal cancer patients (CRC, n=216) after cholecystectomy

Variables	Serum TBA level		p-value
	Low (n=108)	High (n=108)	
Age (years)			
< 50	30 (27.8%)	22 (20.4%)	0.265
≥ 50	78 (72.2%)	86 (79.6%)	
Gender			
Male	57 (52.8%)	62 (57.4%)	0.584
Female	51 (47.2%)	46 (42.6%)	
Tumour location			
Colon	66 (61.1%)	74 (68.5%)	0.319
Rectum	42 (38.9%)	34 (31.5%)	
TNM stage			
I-II	66 (61.1%)	43 (39.8%)	0.003
III-IV	42 (38.9%)	65 (60.2%)	
Pathological grade			
Poorly differentiated	72 (66.7%)	56 (51.9%)	0.038
Moderately to well differentiated	36 (33.3%)	52 (48.1%)	
Lymph node metastasis			
Non-metastasis	67 (62.0%)	46 (42.6%)	0.006
Metastasis	41 (38.0%)	62 (57.4%)	
CA125			
Positive	31 (28.7%)	38 (35.2%)	0.381
Negative	77 (71.3%)	70 (64.8%)	
CA19-9			
Positive	59 (54.6%)	67 (62.0%)	0.334
Negative	49 (45.4%)	41 (38.0%)	
CEA			
Positive	62 (57.4%)	73 (67.6%)	0.159
Negative	46 (42.6%)	35 (32.4%)	

The data are shown as n (percentage). The comparisons of data were done by Fisher's exact test. TBA, total bile acid; CRC, colorectal cancer; TNM, tumour-node-metastasis; CA125, carbohydrate antigen 125; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.

Association of serum TBA levels with tumor progression in CRC patients

To evaluate the relationship between serum TBA levels and clinicopathological characteristics in CRC patients following cholecystectomy, TBA concentrations were compared across TNM stage, histological differentiation, and lymph node status. Patients with advanced TNM stage (III-IV) had significantly higher serum TBA levels ($9.92 \pm 4.58 \mu\text{mol/L}$) than those with early-stage disease (I-II: $7.58 \pm 4.02 \mu\text{mol/L}$; $P < 0.001$) (**Figure 2A**). Similarly, TBA concentrations were higher in patients with moderately to well-differentiated tumours ($10.12 \pm 4.97 \mu\text{mol/L}$) than in those with poorly differentiated tumours ($7.79 \pm 3.80 \mu\text{mol/L}$; $P < 0.001$) (**Figure 2B**). In addition, patients with lymph node metastasis had higher TBA levels ($10.28 \pm 4.56 \mu\text{mol/L}$) than those without lymph node metastasis ($7.34 \pm 3.87 \mu\text{mol/L}$; $P < 0.001$) (**Figure 2C**). These findings show that serum TBA levels differed according to TNM stage, histological differentiation, and lymph node status, although the association with histological differentiation requires further validation.

as the outcome variable and serum TBA levels as the predictor. After adjusting for age, sex, body mass index, diabetes mellitus, hypertension, indication for cholecystectomy, and time since cholecystectomy, serum TBA levels remained associated with CRC status (adjusted odds ratio [OR]=1.32, 95% CI: 1.21-1.44, $P < 0.001$). Within the CRC cohort, serum TBA levels remained associated with advanced TNM stage (adjusted OR=1.18, 95% CI: 1.07-1.30, $P=0.001$) and lymph node metastasis (adjusted OR=1.21, 95% CI: 1.09-1.35, $P < 0.001$) after adjustment for relevant clinical and pathological factors.

Serum TBA levels are associated with FXR expression and tumour-associated macrophages in CRC tissues

To investigate the association between serum TBA levels and FXR expression and TAM-related markers in CRC patients following cholecystectomy, individuals were divided into high and low TBA groups based on the median serum TBA level. Immunohistochemical analysis revealed significantly higher FXR expression in tumour tissues from the high TBA group ($10.35 \pm 4.42\%$) than in the low TBA group ($6.51 \pm 3.03\%$; $P < 0.001$; **Figure 3A, 3B**). Similarly, the

Role of serum total bile acid in colorectal cancer

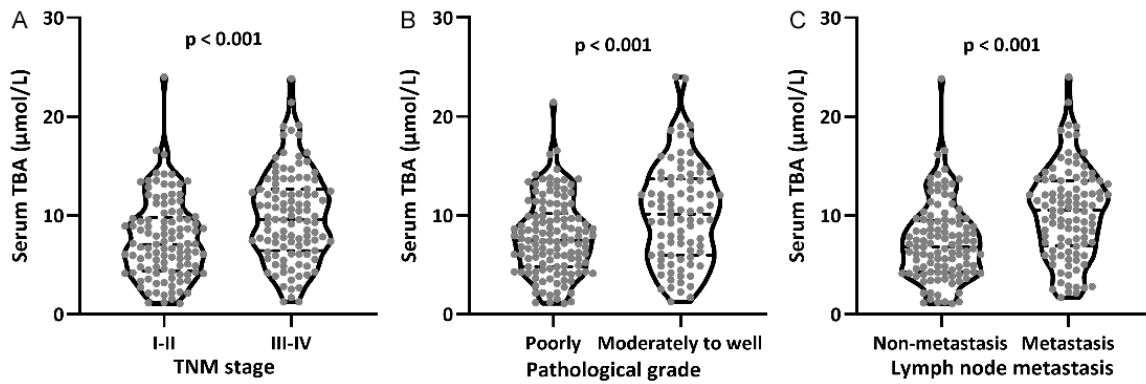


Figure 2. Association of serum TBA concentrations with clinicopathological features in colorectal cancer patients after cholecystectomy. Comparisons of serum TBA levels between TNM stages of I-II and III-IV (A), pathological grades of poorly differentiated and moderately to well differentiated (B), lymph node non-metastasis and metastasis (C) in colorectal cancer patients after cholecystectomy. *P*-values were from an unpaired t-test with Welch's correction.

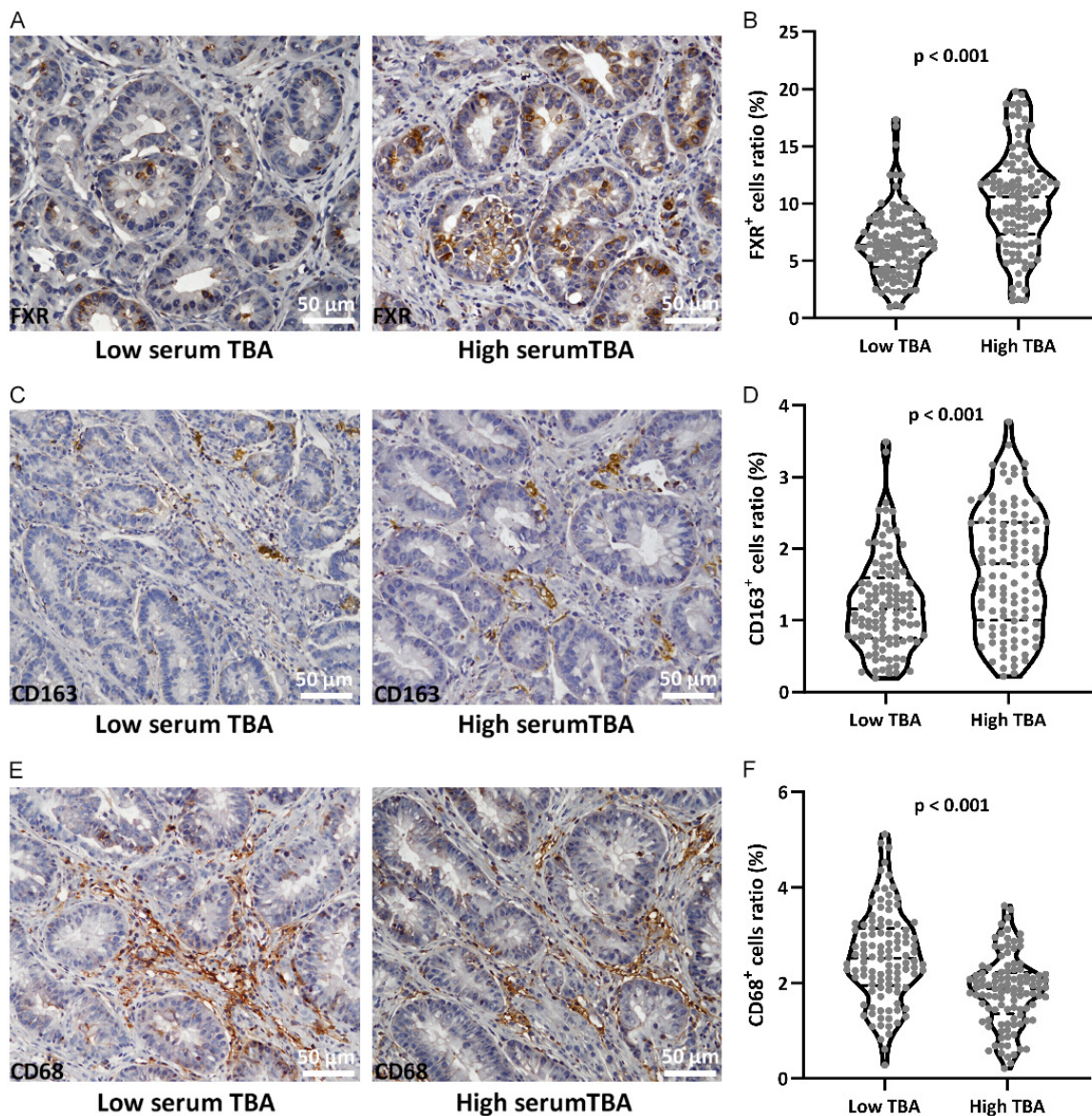


Figure 3. Immunohistochemical expression of the farnesoid X receptor and tumor-associated macrophage markers in colorectal cancer tumors stratified by serum TBA. Based on the median of serum TBA levels in CRC patients after cholecystectomy, patients were divided into the high serum TBA group and the low serum TBA group. Representative IHC staining of FXR (A), CD163 (C), and CD68 (E) from the tumours between the two groups. All micrographs are shown at $\times 400$ magnification with 50 μm scale bars. The quantifications of the FXR positive cells ratio (B), CD163 positive cells ratio (D), and CD68 positive cells ratio (F) between the two groups. *P*-values were from an unpaired *t*-test with Welch's correction.

proportion of CD163⁺ TAMs was significantly higher in the high TBA group than in the low TBA group ($P < 0.001$; **Figure 3C, 3D**). In contrast, the proportion of CD68⁺ TAMs was significantly lower in the high TBA group ($1.84 \pm 0.73\%$) than in the low TBA group ($2.55 \pm 0.95\%$; $P < 0.001$; **Figure 3E, 3F**). Collectively, these findings show that higher serum TBA levels were associated with higher FXR expression and differences in TAM-related marker expression in CRC tissues after cholecystectomy. Multivariable linear regression analyses further showed that serum TBA levels remained significantly associated with FXR, CD163, and CD68 expression after adjustment for TNM stage, tumour differentiation, and lymph node metastasis. No significant multicollinearity was observed among the included variables (all VIF < 2).

Association of serum TBA levels with FXR expression and TAM in colorectal cancer after cholecystectomy

To further examine the relationships among serum TBA levels, FXR expression, and TAM-related markers in CRC patients following cholecystectomy, Pearson correlation analyses were conducted. Serum TBA levels were positively correlated with the proportion of FXR⁺ cells ($r=0.57$, $P < 0.001$, **Figure 4A**). A similar positive correlation was observed between serum TBA levels and the proportion of CD163⁺ TAMs ($r=0.51$, $P < 0.001$; **Figure 4B**), while a negative correlation was observed with the proportion of CD68⁺ TAMs ($r=-0.44$, $P < 0.001$; **Figure 4C**). Furthermore, the proportion of FXR⁺ cells was positively correlated with the proportion of CD163⁺ TAMs ($r=0.48$, $P < 0.001$; **Figure 4D**) and negatively correlated with the proportion of CD68⁺ TAMs ($r=-0.43$, $P < 0.001$; **Figure 4E**). Collectively, these findings show correlations among serum TBA levels, FXR expression, and TAM-related markers in CRC tissues after cholecystectomy.

Discussion

This study demonstrates an association between elevated serum TBA levels and clinico-

pathological and immune features in CRC after cholecystectomy. Elevated TBA levels were associated with CRC status and adverse clinicopathological features, including advanced TNM stage and lymph node metastasis. High TBA concentrations were associated with higher FXR expression and differences in TAM-related markers, including a higher proportion of CD163⁺ TAMs and a lower proportion of CD68⁺ TAMs. Pearson correlation analysis further showed significant correlations among TBA levels, FXR expression, and CD163⁺ and CD68⁺ TAMs. Collectively, these findings provide further evidence of associations among serum TBA levels, FXR expression, and TAM-related immune features in CRC after cholecystectomy.

Previous studies have mainly focused on the role of bile acid metabolism in colorectal cancer development, progression, and prognosis, whereas its relationship with tumour differentiation remains unclear. Secondary bile acids, particularly deoxycholic acid (DCA), have been reported to be associated with colorectal carcinogenesis through pathways involving inflammation, oxidative stress, and epithelial injury [19, 20]. In addition, bile acid signaling pathways, including FXR-mediated regulation, are involved in maintaining intestinal homeostasis and CRC-related biological processes [21]. The association between circulating bile acid levels and tumor differentiation remains unclear. Although serum TBA levels differed across histological differentiation categories in our cohort, this exploratory finding requires further validation.

This study provides evidence of an association between bile acid dysregulation, immune features, and clinicopathological characteristics in CRC following cholecystectomy, highlighting the potential relevance of the association between TBA levels, FXR expression, and TAM-related immune features. Previous studies have reported alterations in bile acid metabolism following cholecystectomy, including changes in second-

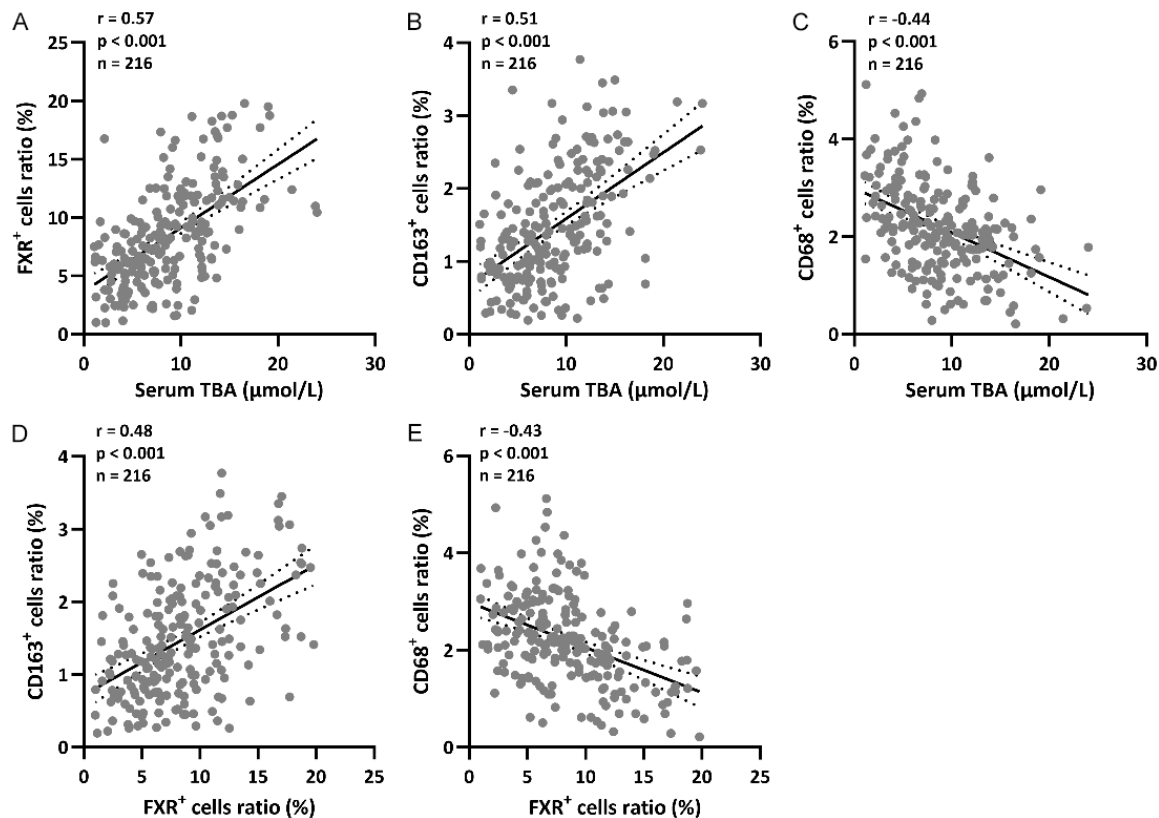


Figure 4. Correlations of serum TBA with farnesoid X receptor and tumor-associated macrophage markers in colorectal cancer tissue after cholecystectomy. Pearson correlation analysis of serum TBA levels with the FXR positive cells ratio (A), CD163 positive cells ratio (B), and CD68 positive cells ratio (C) from the tumor IHC staining in CRC patients after cholecystectomy. Pearson correlation analysis of the FXR positive cells ratio with the CD163 positive cells ratio (D) and CD68 positive cells ratio (E) from the tumor IHC staining in CRC patients after cholecystectomy. Every panel contains $n=216$ observations. The solid line denotes the least-squares linear fit and the shaded band its 95% confidence interval.

ary bile acids such as DCA [9, 22, 23]. Reduced FXR expression has also been reported in CRC tissues [24, 25]. In particular, lower FXR expression has been observed in CRC tissues than in normal or adjacent non-tumor tissues and has been associated with poorer prognosis [25]. However, previous studies mainly investigated FXR dysregulation by comparing CRC tissues with normal or adjacent non-tumor tissues, whereas our study evaluated the association between circulating TBA levels and FXR expression among patients with established CRC after cholecystectomy. Therefore, the increased FXR expression observed in patients with higher TBA levels reflects differences within the CRC population rather than an overall increase in FXR expression in CRC. Previous studies suggest that FXR dysregulation may be associated with changes in the tumor immune microenvironment through metabolic pathways, including modulation of argi-

nine metabolism [26]. Furthermore, secondary bile acids have been reported to impair intestinal barrier integrity and promote epithelial cell proliferation [23]. Our findings extend these observations by showing that, within patients with CRC after cholecystectomy, higher serum TBA levels were associated with higher FXR expression and differences in CD163⁺ and CD68⁺ TAM-related markers. These associations may reflect differences in the tumor immune microenvironment, although their biological basis requires further investigation.

The findings of this study also indicate associations between serum TBA levels and clinicopathological characteristics of CRC following cholecystectomy. The significant associations between elevated TBA levels and key clinicopathological features, including advanced TNM stage and lymph node metastasis, suggest potential clinical relevance, although their utili-

ty for patient assessment remains to be established. Additionally, the observed associations among TBA levels, FXR expression, and TAM-related immune features provide further insight into immune characteristics in post-cholecystectomy CRC. The clinical relevance of these findings requires further validation in larger, independent cohorts.

This study has several limitations. First, it is a single-center retrospective study with a relatively limited sample size, which may limit generalizability and warrants validation in larger, multicenter cohorts. Furthermore, the ROC analysis was performed within the same cohort, and although bootstrap internal validation was conducted, external validation in independent multicenter cohorts is required before clinical application. Second, the study did not include bile acid profiling, such as measurement of specific secondary bile acids, which limited assessment of individual bile acid species. Additionally, the observed correlations require further validation through animal models and mechanistic studies to determine whether causal relationships exist. Although immunohistochemical analysis provided information regarding FXR protein abundance and tissue localization, it does not directly demonstrate FXR activation or downstream transcriptional activity. Therefore, functional studies are needed to further elucidate the relationship between serum TBA levels, FXR expression and TAM-related immune features. In addition, the functional states of immune cells were not assessed. Flow cytometry, transcriptomic analyses, or other functional approaches could provide further information on TAM-related immune features. In addition, CD68 and CD163 were assessed by single-marker immunohistochemistry without dual staining, which limits the assessment of macrophage phenotypes and marker co-expression. Although multivariable analyses were performed to adjust for several potential confounding factors, some variables, such as medication-related information, were not systematically collected due to the retrospective study design and could not be fully evaluated. Therefore, residual confounding cannot be completely excluded. Future multicenter studies incorporating detailed bile acid profiling, immune characterization, and mechanistic analyses are needed to validate and extend these findings.

Conclusion

Our study demonstrates an association between elevated serum TBA levels and clinico-pathological and immune characteristics of CRC following cholecystectomy. High TBA levels were associated with advanced TNM stage and lymph node metastasis. They were also associated with higher FXR expression and differences in TAM-related immune features. These findings provide further evidence of associations among serum TBA levels, FXR expression, and TAM-related immune features in CRC after cholecystectomy.

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All patients signed the informed consent.

Disclosure of conflict of interest

None.

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Role of serum total bile acid in colorectal cancer

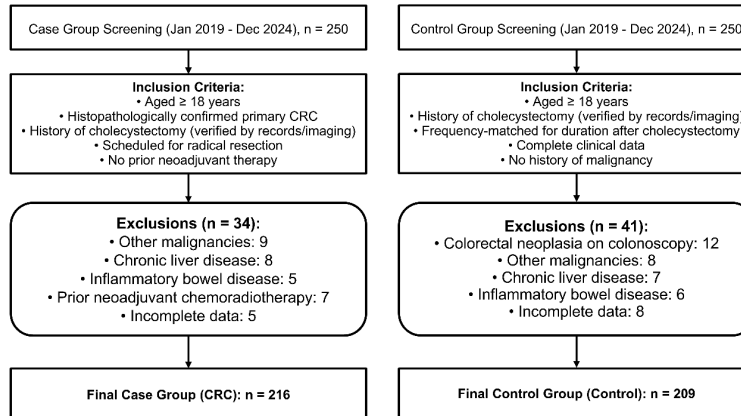


Figure S1. The screening, exclusion, and final inclusion processes for both the case and control groups.