Diagnostic and prognostic values of C-X-C motif chemokine ligand 3 in patients with colon cancer

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Abstract. The diagnostic and prognostic mechanisms of C-X-C motif chemokine ligand 3 (*CXCL3*) in colon cancer (CC) have not yet been reported. Therefore, the objective of the present study was to use cohorts of patients from Guangxi Medical University and the Gene Expression Omnibus (GEO) database to investigate and validate *CXCL3* for the diagnosis and prognosis of CC, and to explore its prospective molecular mechanism. Reverse transcription-quantitative PCR (RT-qPCR) analysis of 38 paired tumor and non-tumor tissues, and immunohistochemistry (IHC) of 212 tumor and 46 non-tumor tissues was conducted to explore the expression of *CXCL3* and its diagnostic and prognostic significance in the Guangxi Medical University CC cohort. A GEO dataset, GSE40967, was used to validate the prognostic significance of *CXCL3*. Gene set enrichment analysis (GSEA) was also conducted to explore the potential molecular mechanisms underlying the effects of *CXCL3* in CC. The RT-qPCR results indicated that *CXCL3* expression was significantly higher in cancer tissues compared with adjacent normal tissues, suggesting that it may have high diagnostic value for CC. Multivariate Cox analysis based on the IHC results suggested that there was no appreciable association between CXCL3 positivity and the overall survival (OS) time of CC. However, a stratified analysis revealed that high expression of CXCL3 was associated with considerably increased mortality in the subgroup of CC patients with tumor size <5 cm (adjusted P=0.042, adjusted HR=2.298, 95% CI=1.030-5.126) and with tumor thrombus (adjusted P=0.019, adjusted HR=5.096, 95% CI=1.306-19.886). In the GSE40967 dataset, high expression of *CXCL3* was closely associated with poor OS in CC (adjusted P=0.049, adjusted HR=1.416, 95% CI=1.002-2.003). Furthermore, GSEA indicated that the high expression of *CXCL3* was closely associated with DNA repair, cell cycle process, cell apoptosis process and the *P53* regulation pathway. In summary, these result suggest that *CXCL3* might serve as a novel biomarker in the diagnosis and prognosis of CC.

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Introduction

Colorectal cancer (CRC) includes malignant tumors that occur in the colon, rectum and anus. With its high morbidity and mortality, CRC is among the most malignant tumors worldwide. It has been estimated that there were >1.8 million new CRC cases and 880,000 CRC-associated deaths in 2018, accounting for approximately one-tenth of all cancer cases and deaths. Among all cancers worldwide, CRC ranks third in terms of morbidity and second in terms of mortality (1). In China, CRC has high incidence and mortality, and is one of the top five most commonly diagnosed tumors (2). The leading cause of mortality for patients with CRC is metastasis. The 5 year overall survival (OS) rate of patients with primary CRC can be as high as 80-90%, but this may be reduced to 5-10% in patients with metastatic tumors (3,4). Like many other cancers, CRC is a heterogeneous disease in which genetic variation, cellular context and environmental effects have an impact on the initiation, progression and metastasis of tumors (5). Accordingly, it is highly crucial to locate biomarkers and prognostic indicators for the early detection of malignant cell transformation.

The use of whole-genome data to screen for markers of tumors, which can be applied to diagnosis and prognosis, is efficient and effective and can be used to guide the exploration of prospective mechanisms. The Gene Expression Omnibus (GEO) is the most comprehensive, well known and largest international public database for the storage and query of expression data; it is developed and maintained by the National Center for Biotechnology Information. Its purpose is to provide a good platform for post-data mining and information promotion by collecting a large amount of high-throughput experimental data (6).

The gene C-X-C motif chemokine ligand 3 (*CXCL3*), a member of the CXC chemokine family, encodes a secreted

growth factor that signals through the G protein-coupled receptor CXC receptor 2, and thereby serves a role in inflammation and acts as a chemoattractant of neutrophils (7,8). Previous studies have investigated the prognostic relationship between *CXCL3* and CRC. The study by Doll *et al* (9) identified no significant correlation between *CXCL3* expression and CRC survival, whereas the findings of Xiong *et al* (10) suggested that CRC patients with high *CXCL3* expression levels had a shorter OS time. More than 50% of CRCs are colon cancer (CC) (1); CC and rectal cancer have different causes (11,12), and their pathogenesis and histological types also differ. In the study conducted by Xiong *et al* (10), patients with colon and rectal cancer from a TCGA dataset were combined for the prognostic analysis of *CXCL3*; however, as patients with colon and rectal cancer are two separate cohorts, the results require further investigation. Furthermore, the study lacked analysis at the protein level. Therefore, the aim of the present study was to use a patient cohort from Guangxi Medical University and a GEO dataset to investigate and validate *CXCL3* for the diagnosis and prognosis of CC, and to explore its prospective molecular mechanism.

Materials and methods

Reverse transcription-quantitative PCR (RT-qPCR) of CXCL3 expression in CC tissue

Patient tissue samples and ethical approval. From April to June 2018, cancer and adjacent normal tissues were continuously collected during the resective surgery of patients with CC in the Department of Colorectal and Anal Surgery, First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi). Immediately after surgery, the tissue was smeared in RNA protection solution and stored in refrigerator at -80˚C. The inclusion criteria for patients were as follows: i) Without restrictions of age and sex; ii) underwent resection of colon tumor; and iii) with a pathological diagnosis of colon cancer. The exclusion criteria include: i) Complicated with other known tumors; ii) received radiotherapy or chemotherapy prior to surgery; iii) refused to provide written informed consent; iv) the tumor was too small for a specimen to be acquired. The study was conducted in accordance with the Declaration of Helsinki, all patients signed an informed consent form, and the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University approved the experimental protocol [Ethics no.: 2019(KY-E-001)].

RNA extraction and RT-qPCR. Total RNA was extracted from the patients' tissues using TRIzol reagent (cat. no. 15596026; Invitrogen, Thermo Fisher Scientific, Inc.). Then, PrimeScript™ RT Reagent kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc.) was used to transform the total RNA into first-strand cDNA. The reverse transcription reaction conditions were as follows: 42˚C for 60 min, 70˚C for 5 min, and 4˚C until required. qPCR was then conducted using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH) in an Applied Biosystems QuantStudio™ 6 Real-PCR System (Thermo Fisher Scientific, Inc.). All procedures were conducted in accordance with the manufacturer's instructions. The expression level of *CXCL3* was calculated using the $2^{\Delta\Delta Cq}$ method (13,14), and was normalized to GAPDH expression. The primer sequences were as follows: *CXCL3* forward, CCA AACCGAAGTCATAGCCAC and reverse, TGCTCCCCT TGTTCAGTATCT; GAPDH forward, GTCAGCCGCATC TTCTTT and reverse, CGCCCAATACGACCAAAT.

Immunohistochemistry (IHC) of CXCL3 expression in CC tissue

Patient tissue samples and ethical approval. Tumor tissue and adjacent normal tissue (slice thickness, 4 μm), fixed with 10% neutral formalin at room temperature for 16 h and embedded in paraffin wax blocks, were retrospectively collected from patients who had undergone colonic tumor resection in the First Affiliated Hospital of Guangxi Medical University between May 2012 and May 2013. The inclusion criteria for patients were as follows: i) Without restrictions of age and sex; ii) received resection of colon tumor; and iii) with a pathological diagnosis of colon cancer. The exclusion criteria include: i) Complicated with other known tumors; ii) received radiotherapy or chemotherapy prior to surgery; iii) refused to sign informed consent; iv) the tumor was too small for a specimen to be acquired. Tumors were identified and categorized according to the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer (8th edition, 2017) (15). Information about the patients was recorded as follows: Sex, age, preoperative carcinoembryonic antigen levels, TNM stage, tumor location, general classification, tumor differentiation, tumor thrombus, tumor size, tumor number, lymph node status, radical resection, tumor metastasis, nerve infiltration and postoperative chemotherapy. The study was conducted in accordance with the Declaration of Helsinki. Prior to the study, all patients received informed consent and ethical approval for the study was provided [Ethics no.: 2019(KY-E-001)].

Evaluation of IHC. IHC was applied for evaluation of the expression of CXCL3. A CXCL3 antibody (cat. no. #35751) supplied by Signalway Antibody LLC, IHC staining reagents (DAB) and Secondary Antibody, HRP (cat. no. D-3004-15) from Shanghai ChangDao Biotech Co., Ltd. were used. Antigen retrieval was conducted using sodium citrate buffer for 2.5 min at high pressure, followed by cooling for 5 min, and washing with PBS buffer for 3 min three times. The IHC procedure and steps were performed strictly following the manufacturers' protocols (incubation with primary antibody incubation at 1:100 dilution, 37˚C for 2.5 h; incubation with ready-to-use secondary antibody for 30 min at room temperature). The slides were observed under an Olympus upright microscope, white light (magnification x400). Two independent pathologists scored the average percentage of positive cells as follows: 0 (0%); 1 (1-25%); 2 (26-50%); 3 (51-75%); and 4 (76-100%). The intensity of staining was scored as follows: 0 (negative); 1 (weak); 2 (moderate) and 3 (strong). The positive cell percentage was multiplied by the staining intensity score as previously described to provide the final pathological score, and a score >2 was considered to indicate a positive staining result (16).

Validation of CXCL3 expression in normal colon and colon tumor tissues. The expression level of *CXCL3* in normal human tissues was obtained from Human Protein Atlas

(HPA: https://www.proteinatlas.org, accessed December 22, 2018) (17). Expression levels of the *CXCL3* gene in normal colon and primary tumor tissues were determined using the online tool GEPIA (http://gepia.cancer-pku.cn/detail.php?gene=cxcl3, accessed February 17, 2019) (18).

Validation cohort for the prognosis value of CXCL3 from the GEO database. A dataset of *CXCL3* gene expression values and corresponding clinical data was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40967, accessed December 23, 2018) (19). The data were chosen according to the following inclusion criteria: i) Histopathological diagnosis of colon cancer; ii) primary tumor that could be surgically removed; iii) complete postoperative follow-up data; iv) all patients did not receive preoperative chemotherapy and/or radiation therapy; and v) number of cases >500. The exclusion criteria include: i) Complicated with other known tumors; ii) the subject of the study was not colon cancer; iii) sample size ≤500. Since these datasets were obtained from public databases, their use did not need require ethical approval.

Gene set enrichment analysis (GSEA). For investigation of the prospective molecular mechanism of *CXCL3* in patients with a prognosis CC, differential metabolic pathways and biological processes at the transcriptome level between high and low *CXCL3* gene expression, which was based on the 75% cut-off values, were analyzed using GSEA (http://software.broadinstitute.org/gsea/index.jsp, accessed December 24, 2018) v3.0 (20). GSEA was used with reference to gene sets from the Molecular Signatures Database, namely c5 GO gene sets for biological process, cellular component and molecular function (c5.bp.v6.2.symbols.gmt, c5.cc.v6.2.symbols.gmt and c5.mf.v6.2.symbols.gmt) and c2 KEGG gene sets (c2.cp.kegg.v6.2.symbols.gmt). The number of permutations was set at 1,000. Enrichment results with one nominal P-value <0.05 and one false discovery rate (FDR) <0.25 were considered statistically significant.

Statistical analysis. The paired t-test was used to analyze the difference in the mRNA expression of *CXCL3* between tumors and adjacent non-tumor tissues. χ^2 test was used to compare the distribution of IHC staining scores between tumors and adjacent non-tumor tissues. The Kaplan-Meier method was performed for survival analysis. Cox proportional hazards regression analysis was applied to calculate the crude and adjusted hazard ratio (HR) and 95% confidence interval (CI) in uni- and multivariate analyses. The FDR in GSEA was adjusted for multiple testing with the Benjamini-Hochberg procedure (21,22). A scatter plot, receiver operating characteristic (ROC) curves and Kaplan-Meier survival curves were drawn using GraphPad Prism 7.0 (GraphPad Software, Inc.). P<0.05 was considered statistically significant. SPSS v.24.0 software (IBM Corp.) was used to conduct the data analysis.

Results

RT-qPCR analysis of CXCL3 expression in CC tissue. RT-qPCR was performed on the CC and adjacent normal tissue samples of 38 patients with CC. These CC patients ranged in age from 35 to 85 years, and included 25 men and 13 women. Analysis using a paired t-test demonstrated that the expression of *CXCL3* in cancer tissues was significantly higher than that in adjacent normal tissues (P=0.0004, 95% CI=0.052-0.164; Fig. 1A and B). In addition, diagnostic ROC curve analysis indicated that *CXCL3* has a high diagnostic value for CC (P<0.0001, AUC=0.896, 95% Cl=0.825-0.967; Fig. 1C).

IHC of CXCL3 expression in CC tissue. IHC testing was performed on another 212 tumor and 46 adjacent normal tissue samples, preserved in wax blocks, that were acquired from 212 patients with CC. The positive signaling of CXCL3, located in the cytoplasm of CC cells or adjacent normal colonic epithelium cells, was shown by the formation of a diffuse brown-yellow or dark-brown color following immunohistochemical staining (Fig. 2). Among the 212 cases of CC, 90 cases were CXCL3-positive (42.5%), while positive CXCL3 expression was observed in only 4/46 (8.7%) of the adjacent normal tissues.

Clinical and pathological factors that may be associated with prognosis were evaluated (Table I). A total of 137 male and 75 female patients, with an average age of 58 years were included in the evaluation. The median follow-up time after surgery was 1,934 days (range, 36-2,236 days); 10 patients were lost to follow-up. The positive rate of CXCL3 in cancer tissues was significantly higher than that in adjacent normal tissues (χ^2=20.536, P<0.001; Fig. 3A) in the 46 CC patients for which both types of tissue were available. The number of patients with IHC scores are shown in Fig. 3B. Diagnostic ROC curve analysis of CXCL3 revealed a moderate diagnostic value for CC (P<0.0001, AUC=0.785, 95% Cl=0.690-0.881; Fig. 3C).

Univariate analysis revealed that advanced TNM stage, poorer tumor differentiation, tumor thrombus, lymph node positivity, non-radical resection and tumor metastasis were associated with poor outcomes (Table I). Kaplan-Meier analysis indicated that CXCL3 expression was not relevant to survival (Fig. 4A) and multivariate analysis showed that CXCL3 positive expression was not relevant to OS following adjustment for TNM stage, tumor differentiation, tumor thrombus and radical resection (adjusted P=0.934, adjusted HR=1.022, 95% CI=0.604-1.729).

Results of the stratified analysis of the association of CXCL3 with OS for different stratified clinical characteristics are displayed in Fig. 5. High expression of CXCL3 was significantly associated with an increased risk of death in the subgroups of patients with tumor size <5 cm (adjusted P=0.042, adjusted HR=2.298, 95% CI=1.030-5.126) and with tumor thrombus (adjusted P=0.019, adjusted HR=5.096, 95% CI=1.306-19.886).

Validation of CXCL3 expression in normal colon and colon tumor tissue. The expression level of *CXCL3* in normal human tissues was obtained from the Human Protein Atlas. Data were extracted from the Functional Annotation of Mammalian Genomes 5 (FANTOM5) project, Genotype-Tissue Expression (GTEx) project and the HPA RNA-seq dataset (Fig. S1). Expression level analysis was performed for the *CXCL3* gene in normal colon and colon tumor tissues. The expression of *CXCL3* in colon cancer tissues was significantly higher compared with that in normal colon tissues (Fig. S2).

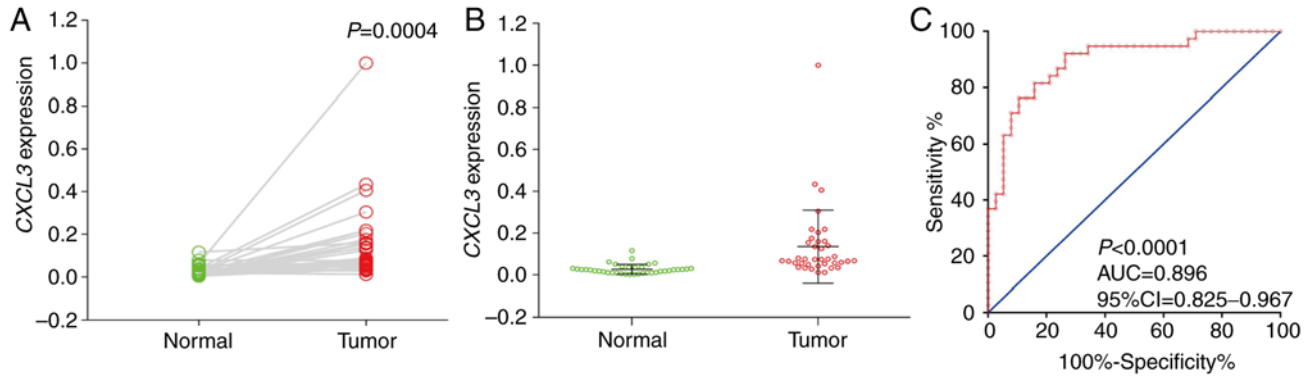

Figure 1. *CXCL3* mRNA expression in 38 paired samples from patients with CC and ROC analysis. (A) Paired comparison of *CXCL3* expression in cancer and adjacent tissues. (B) Comparison of *CXCL3* expression between tumor and normal tissues (the dots represent the expression levels of each sample, and the lines represent the median and quartiles). (C) ROC curve analysis. *CXCL3*, *C-X-C motif chemokine ligand 3*; ROC, receiver operating characteristic; AUC, area under the curve.

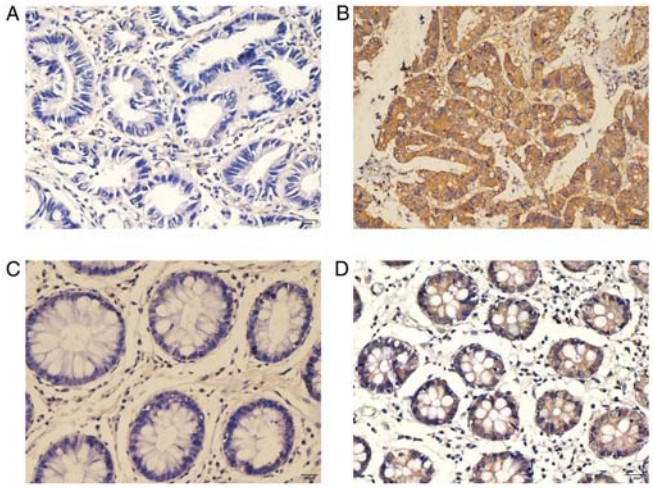

Figure 2. Immunohistochemical staining of *CXCL3* in colon cancer. *CXCL3* signaling was predominantly observed in the cytoplasm the cells. The positive immunohistochemical staining of *CXCL3* was shown as the formation of diffuse brown-yellow or dark-brown color. (A) Negative staining in colon tumor tissue, (B) positive expression in colon tumor tissue, (C) negative staining in adjacent normal tissue and (D) positive expression in adjacent normal tissues. *CXCL3*, C-X-C motif chemokine ligand 3.

Validation of the prognostic value of CXCL3 using the GEO database. The GPL570 expression profile chip data and clinical data were downloaded from the GSE40967 dataset. This included data for 585 patients. The sex, age (years), TNM stage, tumor location, adjuvant chemotherapy, mismatch repair (MMR) status, CpG island methylator phenotype status, chromosomal instability status, *TP53* mutation, *KRAS* mutation, *BRAF* mutation and Cartes d'Identité des Tumeurs (CIT) molecular subtype of these patients were collected.

The patients had a median age of 69 years (range, 22-97 years), and comprised 322 males and 263 females. In the GSE40967 CC cohort, it was observed that age >65 years, advanced TNM stage, *KRAS* mutations and the CIT molecular subtype C4 was associated with a significantly higher risk of CC death (Table II). Kaplan-Meier survival analysis with the 75% cut-off values of *CXCL3* expression suggested that *CXCL3* expression in the GSE40967 cohort was not significantly associated with OS (Fig 4B). However, multivariate analysis indicated that high expression of *CXCL3* (adjusted P=0.049, adjusted HR=1.416, 95% CI=1.002-2.003) was closely associated with poor OS in CC, after adjusting for age, TNM stage, *KRAS* gene and CIT subtypes.

Furthermore, the results of the stratified analysis of the association of *CXCL3* with OS for different stratified characteristics are presented in Fig. 6. High expression of *CXCL3* was associated with a significantly increased risk of death in the following patient subgroups: Age >65 years (adjusted P=0.025, adjusted HR=1.620, 95% CI=1.061-2.473), TNM stage 0-II (adjusted P=0.014, adjusted HR=1.839, 95% CI=1.132-2.989), deficient MMR status (adjusted P=0.014, adjusted HR=3.930, 95% CI=1.319-11.709), *TP53* mutation (adjusted P=0.039, adjusted HR=1.781, 95% CI=1.028-3.085), CIT molecular subtype C4 (adjusted P=0.010, adjusted HR=4.134, 95% CI=1.398-12.219) and male sex (adjusted P=0.026, adjusted HR=1.628, 95% CI=1.059-2.504).

GSEA of CXCL3. GSEA of *CXCL3* was also conducted in the GSE40967 cohort. The genome-wide expression profile dataset of the GSE40967 cohort was assorted into two categories in accordance with the 75% cut-off values of *CXCL3* gene expression. GSEA results of the GSE40967 cohort are displayed in Figs. 7 and 8 and Tables SI and SII, and indicate that the high expression of *CXCL3* exhibited appreciable relevance to DNA repair, cell cycle process, cell apoptosis process and the *P53* regulation pathway.

Discussion

The *CXCL3* gene is located in a cluster of other CXC chemokines on chromosome 4 (23). It is a small cytokine belonging to the CXC chemokine family, and is also known as GRO3 oncogene, GRO protein gamma and macrophage inflammatory protein-2-beta (7,8). CXC chemokines have a heparin-binding domain at the C-terminus of the molecule, that serve different roles in the regulation of angiogenesis (24). Simpson *et al* (25) reported that *CXCL3* is widely expressed in the liver, and is involved in liver injury and inflammation; Luan *et al* (26) reported that *CXCL3* is an important mediator of tumor initiation in human melanoma. Recent studies have shown that *CXCL3* has significant functions in the progression

Table I. Clinical and pathological parameters of 212 patients with colon cancer.

Variable	No. of patients	MST (days)	OS[a], HR (95% CI)[b]	Log rank P-value[c]
Sex				0.801
Male	137	NA	1	
Female	75	NA	0.934 (0.552-1.582)	
Age (years)				0.536
≤65	137	NA	1	
>65	75	NA	1.174 (0.707-1.950)	
CEA (ng/ml)				0.169
1-5	113	NA	1	
>5	93	NA	1.424 (0.858-2.363)	
Missing	6			
TNM stage				<0.0001
I-II	88	NA	1	
III-IV	124	NA	5.049 (2.563-9.945)	
Location				0.806
Right	102	NA	1	
Left	109	NA	0.929 (0.565-1.529)	
Both	1	NA	0 (0-2.209x10^{211})	
General classification				0.691
Invasive	11	NA	1	
Ulcerative	153	NA	1.511 (0.367-6.221)	
Mass	42	NA	1.203 (0.267-5.428)	
Missing	6			
Tumor differentiation				0.019
Well	10	NA	1	
Moderately	160	NA	1.451 (0.352-5.993)	
Poor	42	NA	3.076 (0.710-13.318)	
Tumor thrombus				<0.0001
No	185	NA	1	
Yes	26	660	4.571 (2.568-8.134)	
Missing	1			
Tumor size (cm)				0.236
<5	90	NA	1	
≥5	116	NA	0.739 (0.447-1.221)	
Missing	6			
Tumor number				0.138
One	205	NA	1	
Two	7	1,917	2.119 (0.768-5.844)	
Lymph node				<0.0001
Negative	120	NA	1	
Positive	91	NA	3.546 (2.075-6.061)	
Missing	1			
Radical resection				<0.0001
Yes	175	NA	1	
No	37	481	11.536 (6.836-19.469)	
Tumor metastasis				<0.0001
No	179	NA	1	
Yes	33	401	14.344 (8.376-24.565)	
Nerve infiltration				0.173
No	207	NA	1	
Yes	4	1,079	2.572 (0.628-10.540)	
Missing	1			

Table I. Continued.

Variable	No. of patients	MST (days)	OS[a], HR (95% CI)[b]	Log rank P-value[c]
Postoperative chemotherapy				0.833
No	69	NA	1	
Yes	124	NA	1.061 (0.610-1.846)	
Missing	19			
CXCL3				0.730
Negative	122	NA	1	
Positive	90	NA	0.914(0.548-1.524)	

[a]OS times were available for 202 patients; [b]HR and 95% CI values were calculated using a univariate Cox proportional hazards regression model; [c]P-values were calculated by the Kaplan-Meier method with a log-rank test. MST, median survival time; OS, overall survival time; TNM, tumor node metastasis; CEA, carcinoembryonic antigen; *CXCL3*, C-X-C motif chemokine ligand 3; NA, not available.

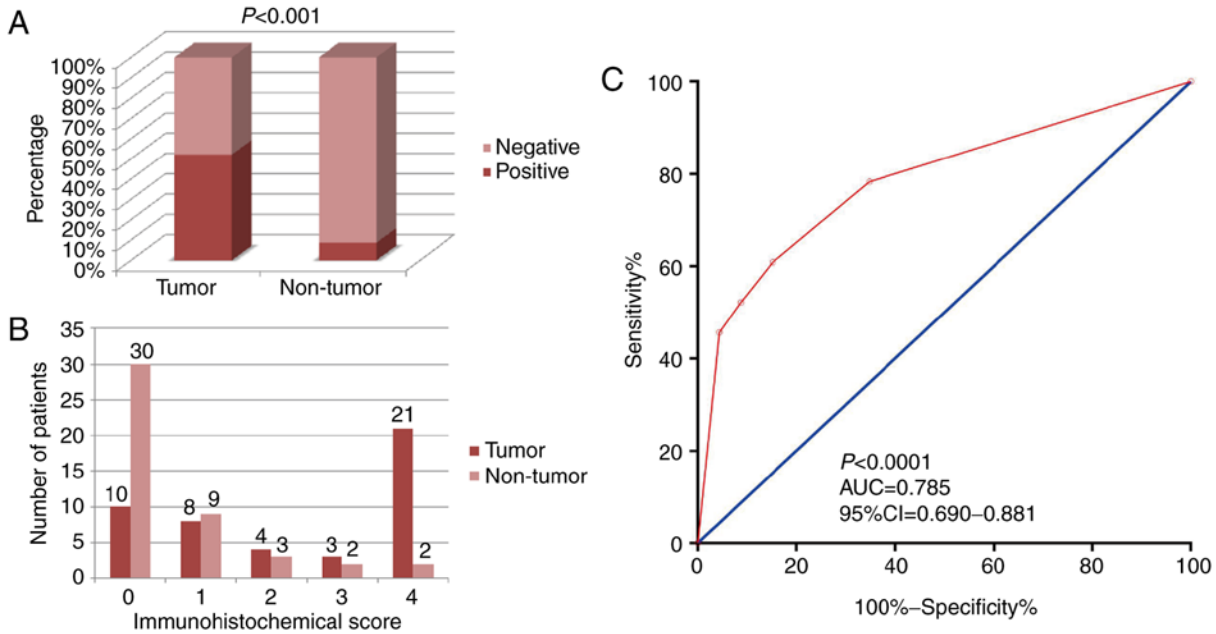

Figure 3. Immunohistochemical staining scores for *CXCL3* in 46 pairs of tumor and adjacent normal tissues from patients with colon cancer, and ROC curve analysis. (A) Stacked column chart showing the positive and negative staining percentages of cancer and adjacent normal tissues. (B) Graph showing the immunohistochemical score distribution of *CXCL3*. (C) ROC curve analysis. *CXCL3*, C-X-C motif chemokine ligand 3; ROC, receiver operating characteristic; AUC, area under the curve.

and metastasis of malignant tumors. See *et al* (27) reported that *CXCL3* is involved in breast cancer metastasis and may be a potential target for cancer treatment. Gui *et al* (28) suggested that *CXCL3* is overexpressed in prostate cancer and might play various roles in prostate cancer progression and metastasis. However, Li *et al* (29) found no significant difference in CXCL3 expression in non- and low-metastatic colon cancer cells, compared with highly metastatic colon cancer cells. Furthermore, Farquharson *et al* (30) demonstrated that insulin and adiponectin can participate in the occurrence of colon cancer through the regulation of CXCL3.

The study by Doll *et al* (9) showed that when *CXCL3* mRNA expression was tested by RT-qPCR in the CRC tissues of 97 patients and normal colon tissues of 16 patients, *CXCL3* gene expression was significantly increased in CRC compared with normal colon tissue. In the study by Xiong *et al* (10), the analysis of 695 RNA results from 645 CRC patients from the TCGA showed that the expression of *CXCL3* in cancer tissues was considerably higher than that in adjacent normal tissues, which was verified by the RT-qPCR testing of 25 pairs of fresh CRC and adjacent noncancerous tissues collected from 25 patients at the First Affiliated Hospital of Chongqing Medical University. Similar results have also been found in other cancer studies; for example, one study found that *CXCL3* was higher in early stage non-small cell lung cancer tissue as compared with the matched normal tissue (31). A meta-analysis also obtained comparable results for *CXCL3* in breast cancer (27). In the present study, the analysis of *CXCl3* mRNA in the paired cancer and adjacent tissues of 38 CC patients revealed that *CXCl3* was overexpressed in CC; the IHC scores of cancer and adjacent normal tissues in 46 patients revealed that the *CXCL3* score for cancer tissues were higher than that

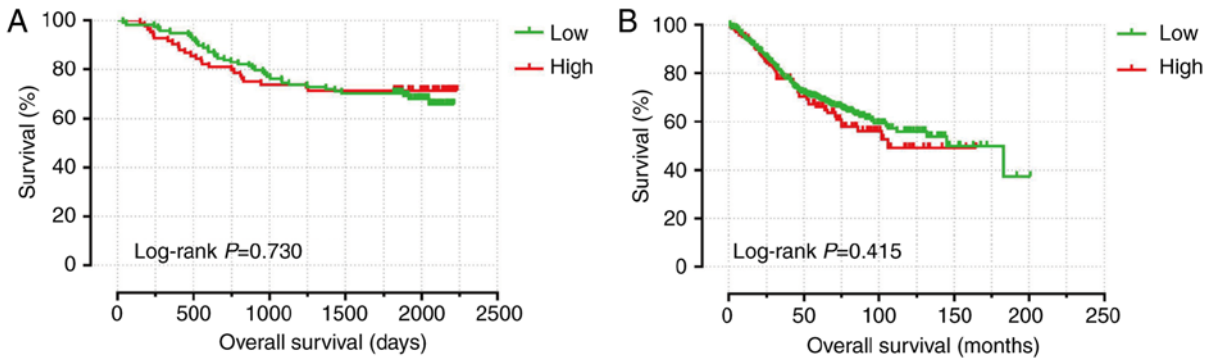

Figure 4. Kaplan-Meier curves for the survival of colon cancer patients with high and low *CXCL3* gene expression levels. (A) Kaplan-Meier curve for the patient cohort from Guangxi Medical University (May 2012 to May 2013) and (B) Kaplan-Meier curve for the GSE40967 cohort. *CXCL3*, C-X-C motif chemokine ligand 3.

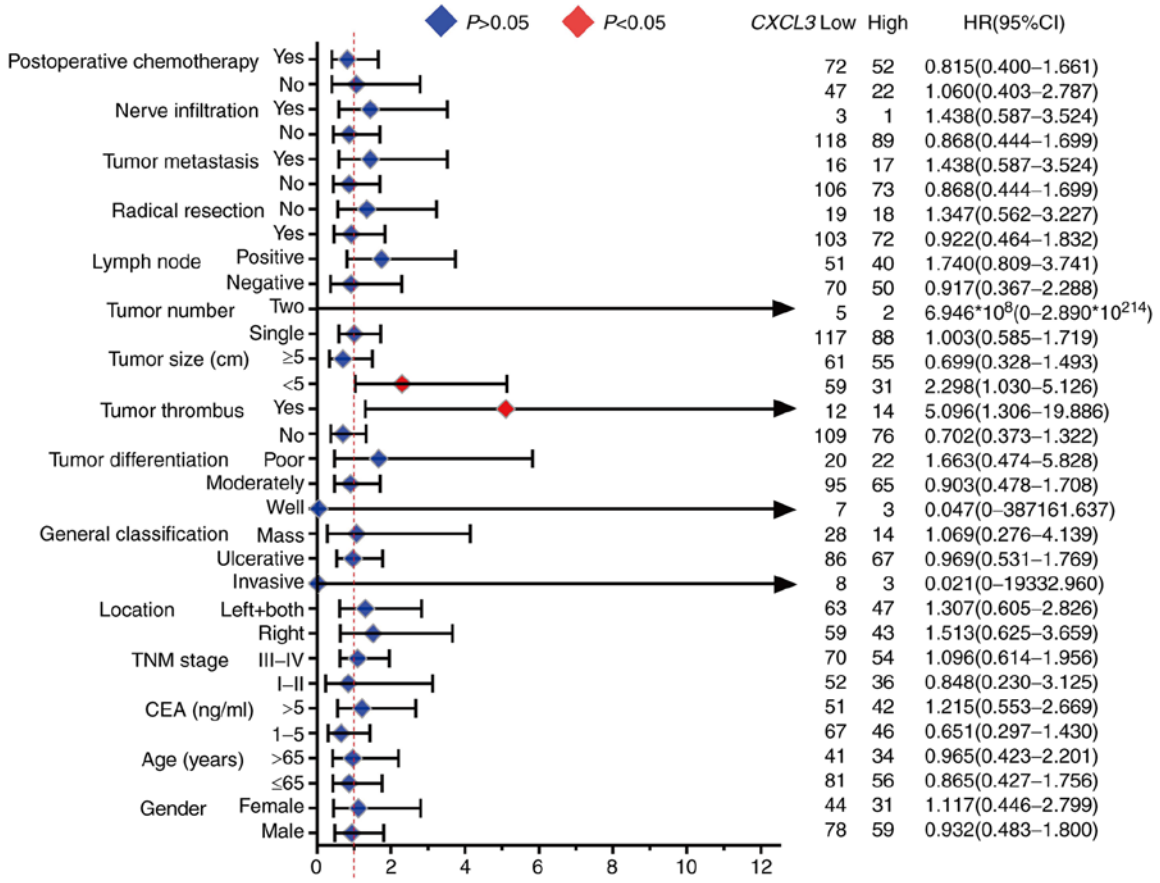

Figure 5. Forest plot of the stratified analysis of the association of *CXCL3* with OS for different characteristics in the colon cancer patient cohort from Guangxi Medical University. *CXCL3*, C-X-C motif chemokine ligand 3; TNM, tumor node metastasis; CEA, carcinoembryonic antigen.

for the adjacent tissues. These results are consistent with the results obtained using GEPIA. Therefore, the present study verified the overexpression of *CXCL3* in CC tissues at both the genetic and protein levels, which indicates that *CXCL3* may be a potential marker for the diagnosis of CC.

Previous studies have found that overexpression of *CXCL3* indicates poor prognosis, Specifically, hepatocellular carcinoma patients with higher *CXCL3* expression have been observed to have a shorter survival time (32). In addition, shorter OS was observed in CRC patients with increased *CXCL3* expression (10). In the current study of CC, similar results were obtained. In the multivariate analysis of the Guangxi Medical University cohort of 212 CC patients, although *CXCL3* expression was not closely and directly connected with OS time, further subgroup analysis revealed that *CXCL3* positive expression in patients who had a tumor diameter <5 cm or a tumor embolus indicated poorer prognosis. A subsequent multivariate analysis of prognosis in the GEO cohort, which was performed to verify the results obtained from Guangxi Medical university cohort, found that *CXCL3* gene expression was notably relevant to overall patient survival, and patients with high *CXCL3* gene expression had

Table II. Clinical and pathological parameters of 585 patients with colon cancer from the GSE40967 cohort.

Variable	No. of patients	MST (months)	OS[a], HR (95% CI)[b]	Log-rank P-value[c]
Sex				0.066
Male	322	112	1	
Female	263	183	0.765 (0.573-1.020)	
Age (years)				0.010
≤65	228	NA	1	
>65	356	105	1.479 (1.094-1.999)	
Missing	1			
TNM stage				<0.0001
0-II	313	183	1	
III-IV	270	105	1.774 (1.335-2.358)	
Missing	2			
Location				0.584
Distal	351	145	1	
Proximal	232	NA	1.084 (0.812-1.447)	
Missing	2			
Chemotherapy adjuvant				0.607
No	326	183	1	
Yes	240	145	0.926 (0.690-1.243)	
Missing	19			
MMR status				0.397
dMMR	77	NA	1	
pMMR	459	NA	1.227 (0.762-1.977)	
Missing	49			
CIMP status				0.589
Negative	420	145	1	
Positive	93	NA	1.115 (0.751-1.656)	
Missing	72			
CIN status				0.170
Negative	112	NA	1	
Positive	369	145	0.770 (0.529-1.121)	
Missing	104			
TP53 mutation				0.312
Mutant	190	105	1	
Wild type	161	NA	0.836 (0.590-1.185)	
Missing	234			
KRAS mutation				
Mutant	217	132	1	0.037
Wild type	328	145	0.736 (0.551-0.983)	
Missing	40			
BRAF mutation				0.689
M	51	NA	1	
WT	460	145	0.900 (0.538-1.508)	
Missing	74			
CIT molecular subtype				0.002
C1	116	86	1	
C2	104	NA	0.722 (0.447-1.165)	
C3	74	NA	0.639 (0.360-1.137)	
C4	59	46	1.790 (1.125-2.850)	
C5	152	145	0.855 (0.567-1.288)	
C6	60	105	1.001 (0.602-1.665)	
Missing	20			

Table II. Continued.

Variable	No. of patients	MST (months)	OS[a], HR (95% CI)[b]	Log-rank P-value[c]
CXCL3				0.415
Low	439	145	1	
High	146	106	1.139 (0.829-1.566)	

[a]OS times were available for 579 patients; [b]HR and 95% CI values were calculated using a univariate Cox proportional hazards regression model; [c]log-rank P-values were calculated by the Kaplan-Meier method with a log-rank test. MST, median survival time; OS, overall survival time; TNM, tumor node metastasis; MMR, mismatch repair; dMMR deficient mismatch repair; pMMR, proficient mismatch repair; CIMP, CpG island methylator phenotype; CIN chromosomal instability; CIT, Cartes d'Identité des Tumeurs; CXCL3, C-X-C motif chemokine ligand 3.

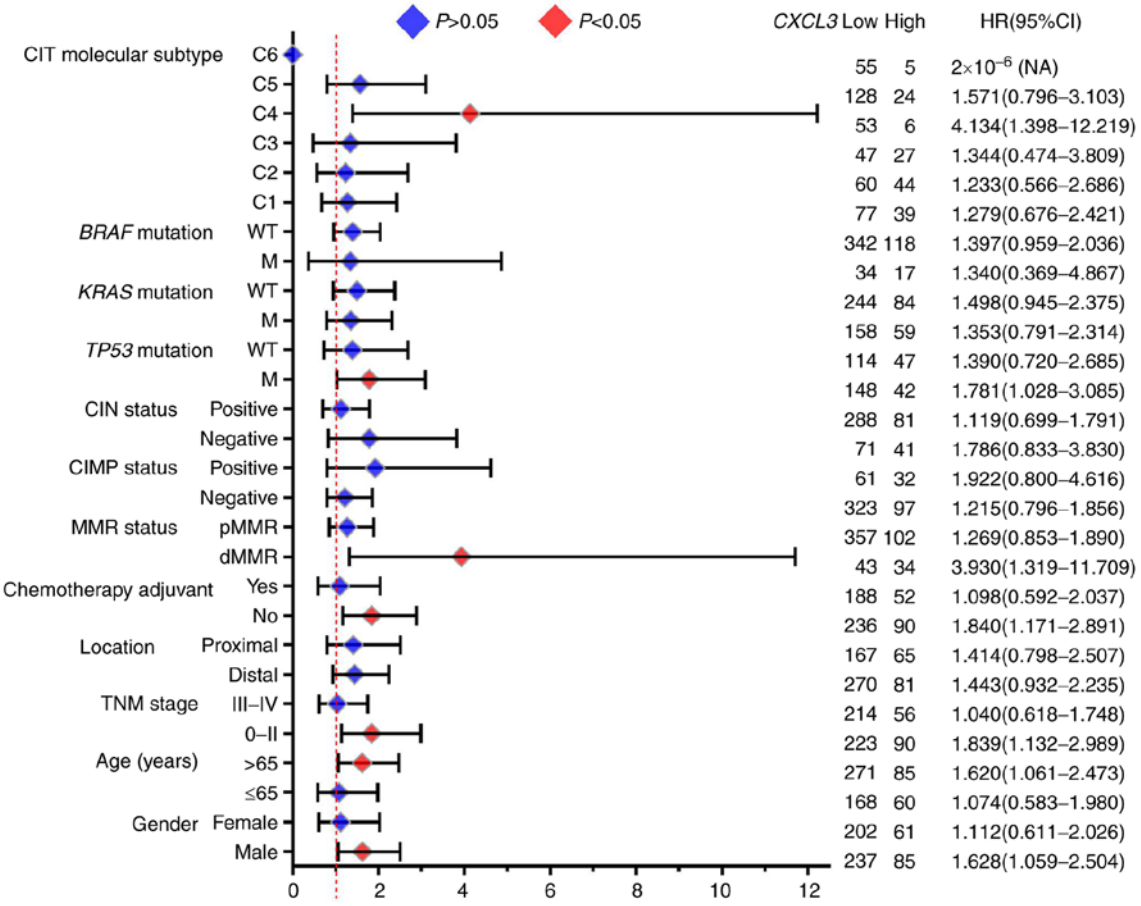

Figure 6. Forest plot of the stratified analysis of the association of *CXCL3* with OS for different characteristics for the GSE40967 cohort. *CXCL3*, C-X-C motif chemokine ligand 3; CIT, Cartes d'Identité des Tumeurs; CIN, chromosomal instability; CIMP, CpG island methylator phenotype; MMR, mismatch repair; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair; TNM, tumor node metastasis.

shorter survival times. These results also suggest that *CXCL3* might be a candidate prognostic biomarker for CC.

CXCL3 is considered to serve a major role in tumor initiation and invasion. The expression of *CXCL3* in normal colon tissue is high, indicating that it plays a certain role in the physiological function of normal intestinal tissues, but is dysregulated in cancer, indicating that expression disorder of *CXCL3* may be involved in the tumorigenesis of CC (33). To examine the potential mechanism of *CXCL3* in CC, a genome-wide RNA sequencing dataset in GSEA was analyzed in the present study. The results indicated that the mechanism by which *CXCL3* affects CC prognosis may involve biological processes and signaling pathways connected with DNA repair, cell cycle, apoptosis and *P53* signaling. Previous studies have suggested an association between DNA repair and CRC development (34-36). Soreide *et al* (37) reported that cell cycle and apoptosis are associated with the prognosis of CRC. Numerous studies have reported a relationship between *P53* and the development of CRC (38-40). However, to the best of our knowledge, the functional correlations of DNA repair, cell

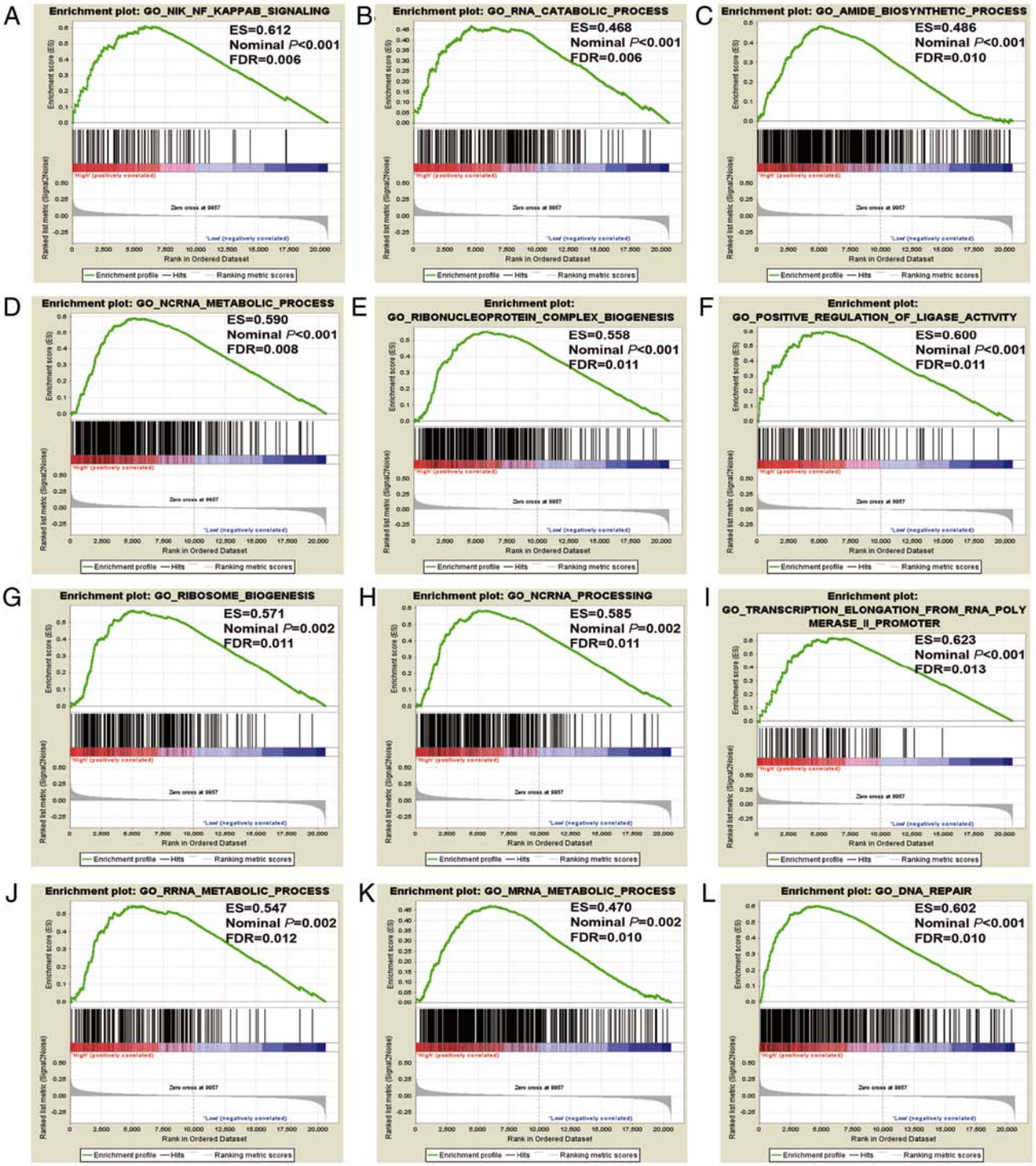

Figure 7. GSEA results of *CXCL3* in the GSE40967 cohort, based on a GO dataset. (A) NIK NF κB signaling, (B) RNA catabolic process, (C) amide biosynthetic process (D) ncRNA metabolic process, (E) ribonucleoprotein complex biogenesis, (F) positive regulation of ligase activity, (G) ribosome biogenesis, (H) ncRNA processing, (I) transcription elongation from RNA polymerase II promoter, (J) rRNA metabolic process, (K) mRNA metabolic process and (L) DNA repair. GSEA, gene set enrichment analysis; *CXCL3*, C-X-C motif chemokine ligand 3; ES, enrichment score; FDR, false discovery rate; ncRNA, non-coding RNA; rRNA, ribosomal RNA; mRNA, messenger RNA.

cycle, apoptosis and *P53* with *CXCL3* have not been previously reported. The GSEA of *CXCL3* in the present study supported the conclusion that *CXCL3* might affect CC via DNA repair, cell cycle, apoptosis and the *P53* pathway. However, these hypotheses require further research for confirmation.

The present study used GSE40967 and Guangxi cohorts to analyze the prognostic value of CXCL3 in CC at the mRNA and protein levels. These two cohorts belong to retrospective cohort studies with a level of evidence of four, as defined on the basis of the Oxford Centre for Evidence-based Medicine-Levels of Evidence (41). However, the present study has certain limitations. The clinical information from the GEO database was incomplete, and information such as tumor size, histology, tumor differentiation, lymphatic invasion and venous invasion were unattainable

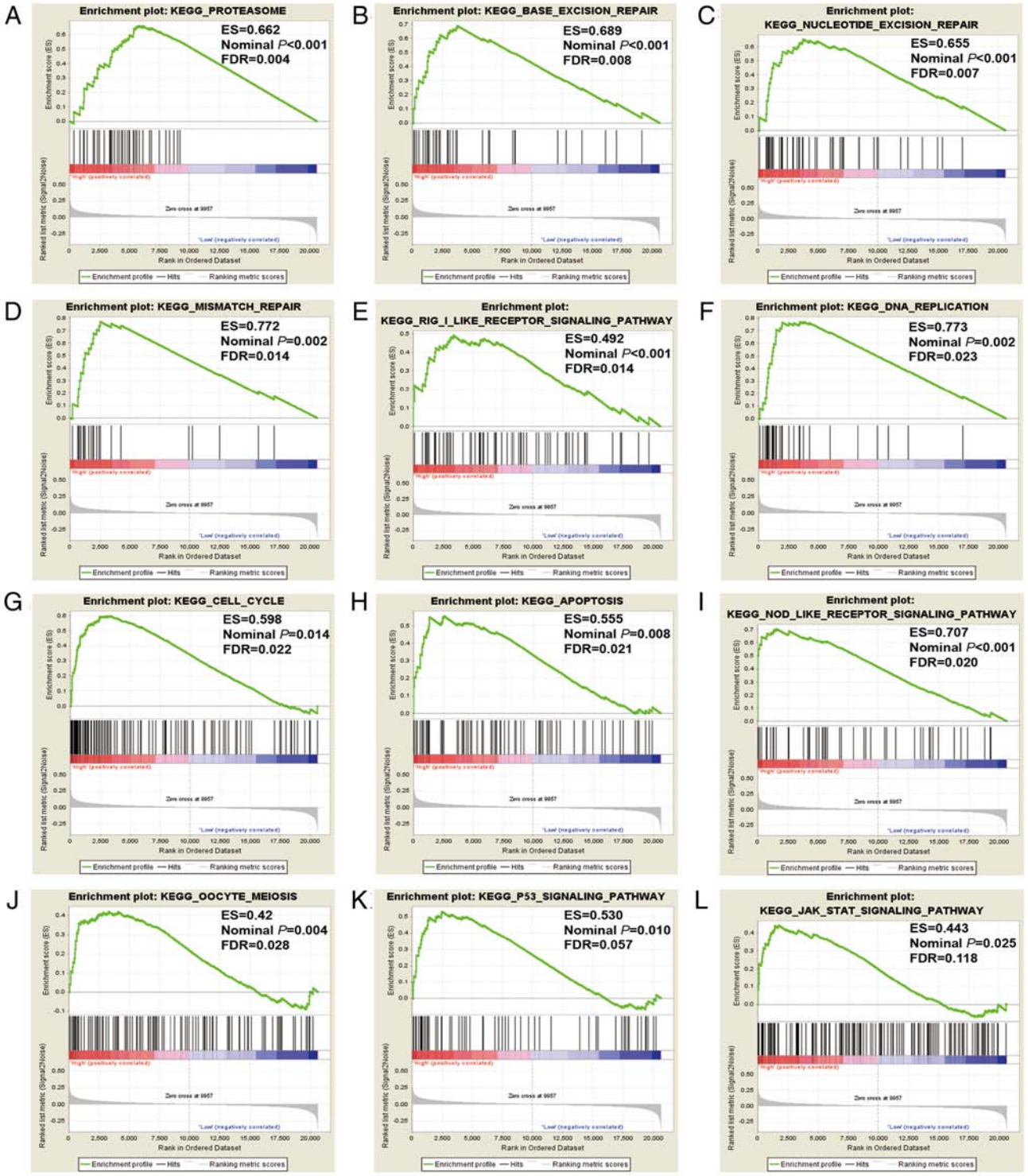

Figure 8. GSEA results of *CXCL3* in the GSE40967 cohort, based on a KEGG dataset. (A) proteasome, (B) base excision repair, (C) nucleotide excision repair, (D) mismatch repair, (E) RIG I-like receptor signaling pathway, (F) DNA replication, (G) cell cycle, (H) apoptosis, (I) NOD-like receptor signaling pathway, (J) oocyte meiosis, (K) P53 signaling pathway, (L) JAK STAT signaling pathway. GSEA, gene set enrichment analysis; *CXCL3*, C-X-C motif chemokine ligand 3; RIG I, retinoic acid-inducible gene-I; NOD, nucleotide-binding oligomerization domain; ES, enrichment score; FDR, false discovery rate.

from the GEO website. The results of this study also require validation in a larger sample population and in a multi-center, multi-regional and multi-ethnic population. Furthermore, *in vitro* and *in vivo* functional trials are needed to further explore the roles of *CXCL3* in CC initiation, development, metastasis, proliferation and angiogenesis. However, to the best of our knowledge, the current study is the first to discover the value of *CXCL3* in the diagnosis and prognosis of CC, rather than CRC. Another advantage of this study is that, in addition to identifying the prognostic value of *CXCL3* in CC in large samples, a GEO genome-wide dataset was also used to explore prospective molecular mechanisms through the GSEA approach.

In conclusion, the present study demonstrated that *CXCL3* is not only considerably upregulated in tumor tissue but also has

potential diagnostic value in patients with CC. Survival analysis in Guangxi Medical University and GEO cohorts suggested that *CXCL3* may serve as a potential prognostic biomarker in CC. The prospective molecular mechanism identified by GSEA suggested that *CXCL3* may influence the prognosis of CC through involvement in the regulation of DNA repair, cell cycle process, cell apoptosis process and *P53* regulation pathways. However, these results require further verification using *in vivo* and *in vitro* experiments in future studies.

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Availability of data and materials

The analyzed datasets generated during the study are available from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/), and the datasets for the colon cancer cohort from the First Affiliated Hospital of Guangxi Medical University used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GTR and YZG wrote the manuscript. GTR and FG made substantial contributions to the conception, design and intellectual content of the study. GTR, YZG, XWL, SW, WH, XKW, GZZ and CL made key contributions to the analysis and interpretation of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients signed an informed consent form, and the experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University [No. 2019(KY-E-001)].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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