Iran J Public Health, Vol. 53, No.9, Sep 2024, pp.2079-2089

Original Article

The Effect of C-X-C Motif Chemokine Ligand 12 in Colorectal Cancer Associated with Chemoresistance and Radioresistance as Well as Stemness

Wuzhen Dong [1], ***Wen Lin*** [2], ****Chong Li*** [3]

1. *Department of Proctology, Jinhua Central Hospital, Jinhua, Zhejiang, China*
2. *Department of Nursing, Ziyang College of Dental Technology, Ziyang, Sichuan, China*
3. *Department of General Surgery, The Third Affiliated Hospital of Chongqing Medical University, Shuanghu Branch Road No. 1, Yubei District, Chongqing, China*

***Corresponding Author:** Email: waiwailaoli@cqmu.edu.cn

(Received 19 Feb 2024; accepted 5 Apr 2024)

Abstract

Background: We aimed to explore the role of C-X-C motif chemokine ligand 12 (*CXCL12)* and cytokine-cytokine receptor interaction signaling pathway in the radiotherapy and chemotherapy resistance as well as cell stemness in colorectal cancer (CRC).

Methods: Bioinformatics analysis was used to identify the differentially expressed mRNAs and signal pathways closely related to differentially expressed mRNAs have also been analyzed in March 2022 at the Jinhua Central Hospital, China. Then, the expression of *CXCL12* was detected by qRT-PCR in colorectal cancer cells and testing the effects of transfecting *CXCL12* into different CRC-derived cell lines. The effects of *CXCL12* on cell proliferation were evaluated by chemosensitivity assay and radiation sensitivity assay.

Results: Bioinformatics analysis of DEGs found a total of 2429 differentially expressed genes, *THBS3* and *CXCL12* genes are two abnormally highly expressed genes in the CRC. KEGG analysis showed the correlative signaling pathway, cytokine-cytokine receptor interaction, which is related to cell stemness. Furthermore, the expression of *CXCL12* in CRC cells was detected and an increasing trend was obtained in CRC cells. In addition, the chemosensitivity and radiotherapy tolerance were elevated after transfected with *CXCL12*.

Conclusion: CXCL12 could be a potential promote biomarkers in CRC and also promote the chemosensitivity and radiotherapy tolerance.

Keywords: Colorectal cancer (CRC); *C-X-C motif chemokine ligand 12 (CXCL12)*; Chemosensitivity; Radiation-sensitivity; Stemness

Introduction

Colorectal cancer (CRC) is highly aggressive, and its incidence and mortality rate are among the top 10 tumors in the world (1, 2). Risk factors for CRC include age, gender, diet and other lifestyle habits (3). The treatment methods of CRC include surgery, radiotherapy and chemotherapy. The systemic treatment of CRC patients has made great progress in the past ten years (4). Radiotherapy was utilized combination with chemotherapy, during before or after surgical resection

to reduce the risk of recurrence, however it usually has a good response at the beginning, but most patients still experience radiotherapy resistance (RT) (5). In addition, colonoscopy is often performed for symptomatic patients, but it is worth noting that the value of symptoms as an indicator of colorectal cancer is low, and accurate diagnosis can enable CRC patients to receive timely treatment (6). Therefore, it is a more breakthrough idea to determine the internal molecular mechanism of CRC progression and significant pathways for CRC radiotherapy and chemotherapy.

The core origin of tumor development is a small subset of cancer cells (called cancer stem cells, CSCs), with the characteristic of self-renewal through cell division to form daughter-cells and differentiation, which have tumor initiation capabilities (7, 8). CSCs of solid tumors were first found in breast cancer, and subsequently confirmed in ovarian cancer, prostate cancer, pancreatic cancer, liver cancer and colorectal cancer, and their specific markers were explored (9-11). Yamashita et al used CD133 and EpCAM markers to define stem cells (CSC) in liver cancer, and explored the relationship with tumor progression, drug resistance, metastasis and recurrence (12). Paolillo *et al.* have established models for studying cell metastasis, adhesion, and drug resistance by culturing stem cell-like cancer cells (13). In addition, TUSC3 promotes the formation of cellular stemness and induces drug resistance via Hedgehog signaling pathway in CRC (14). However, there are still a lot of blanks in the biological functions related to CRC, which need to be explored.

In present study, we aimed to explore the role of C-X-C motif chemokine ligand 12 (CXCL12) and cytokine-cytokine receptor interaction signaling pathway in the radiotherapy and chemotherapy resistance as well as cell stemness in CRC.

Methods

CRC mRNA dataset GSE15781 was acquired from the GEO database (https://www.ncbi.nlm.nih. gov/geo/) in March 2022 at the Jinhua Central Hospital, China. The expression of mRNAs were downloaded from nine tumor irradiated samples and thirteen tumor samples. Those extremely lowly and highly expressed genes in mRNA data were filtered out by R package normalization function.

Differential expression genes (DEGs) analysis

DEGs (mRNAs) were selected based on their fold change and adjusted p-values. We performed the DEGs with threshold parameters defned as $|\log2FC|>1$ and FDR<0.05 (FC: fold change, FDR: false discovery rate), which were generated by the DESeq package from R package. The differentially expressed mRNAs identified were visualized using volcano plots and pheatmap.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

Highly functionality pathway enrichment was executed by KEGG analysis. To better comprehend the mechanisms of KEGG (http://www.kegg.jp/) pathway analysis of DEGs was conducted using the clusterProfiler package. KEGG analysis is used to interpret the potential functions and pathways and the threshold was set as $P < 0.05$.

Cell culture

Three cell lines, including colorectal cancer cell lines LoVo (culture condition:F-12K+10% FBS) and SW480 (culture condition:DMEM-H+10% FBS), and normal colorectal mucosal cells FHC (culture condition: RPMI-1640+10% FBS), were purchased from ATCC (Shanghai, China). The incubator humidifed settings: 5% CO_2, 37 ˚C.

Vector construction

The CDS sequence of *CXCL12* gene was first obtained, and the expression vector was selected according to the experimental needs. Using FuGENE® 6 transfection reagent, the detailed steps were performed according to the manufacturer's protocol, and *CXCL12* overexpression vectors were transfected in SW480 and LoVo cell lines, respectively, while empty vectors were set as controls.

Cell treatment and schedule

Chemosensitivity assay: sensitivity of SW480 and LoVo cells to fluorouracil (5-FU, chemotherapy drug) was tested, specific steps were briefly described as follows: a 96-well plate was used to inoculate cells at a concentration of 2000 cells/well, and the culture conditions: 5% CO_2, 37°C. The fresh medium was replaced after 24 h and 5-FU was administered at the predetermined IC50 concentration according to the instructions. The cell viability and proliferation were assayed by CCK8 assay at the time point of chemotherapy treatment 24 and 48 h.

Radiation sensitivity assay: Radiation sensitivity of SW480 and LoVo Cells were measured, briefly described as follows: a 96-well plate was used to inoculate cells at a concentration of 2000 cells/well, and the culture conditions: 5% CO_2, 37°C. Gamma Cell 40 Exactor (MDS Nordion, Ontario, Canada) irradiation was performed on CRC cell lines that had been cultured for 24 hours. Cell viability was measured by CCK8 assay, 24 and 72 h after the cells were treated with radiation therapy.

Isolation of total RNA and quantitative RT-PCR

Total RNA from the CRC tumor samples and cell lines was extracted using the RNeasy Mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. mRNA expression levels were measured by qRT-PCR using SYBR® Premix Ex Taq™ (Takara). The cycling conditions were set as follows: 95°C (30 s)→[95°C (5 s), annealing (5 s), 72°C (30 s)]*40 cycles→72°C (5 min). 2-ΔΔCT method was hired for measuring fold change and internal reference gene was: GAPDH. The primers sequence was displayed in Table 1.

Table 1: Sequences of primers for qRT-PCR

Primer name	*Sequence(5' to 3')*
CXCL12 forward	GCTGGCACCCCTGGTCCAGGT
CXCL12 reverse	CCAGGCCCAGGCGGCGACCCAGCTTTCTT
GAPDH forward	GCTCCCTCTTTCTTTGCAGCAAT
GAPDH reverse	TACCATGAGTCCTTCCACGATAC

Statistical analysis

Experimental results collation and data analysis with the help of two statistical software, SPSS 26.0 (IBM Corporation, Armonk, USA) and GraphPad (GraphPad Software, San Diego, USA). Student 's *t*-test (two-tailed), Kruskal-Wallis test and Spearman correlation test were used as the main statistical evaluation methods and $P<0.05$ indicates statistical significance. All values are expressed as means ± SD.

Results

Differentially expressed genes (DEGs) analyses

In order to identify the differentially expressed mRNAs between colorectal cancer samples and tumour irradiated samples, DEGs detection were performed using the R packages and visualized by pheatmap (Fig. 1A). Bioinformatics analysis of DEGs found a total of 2429 differentially expressed genes, and the pheatmap showed the top 10 genes with high and low expression.

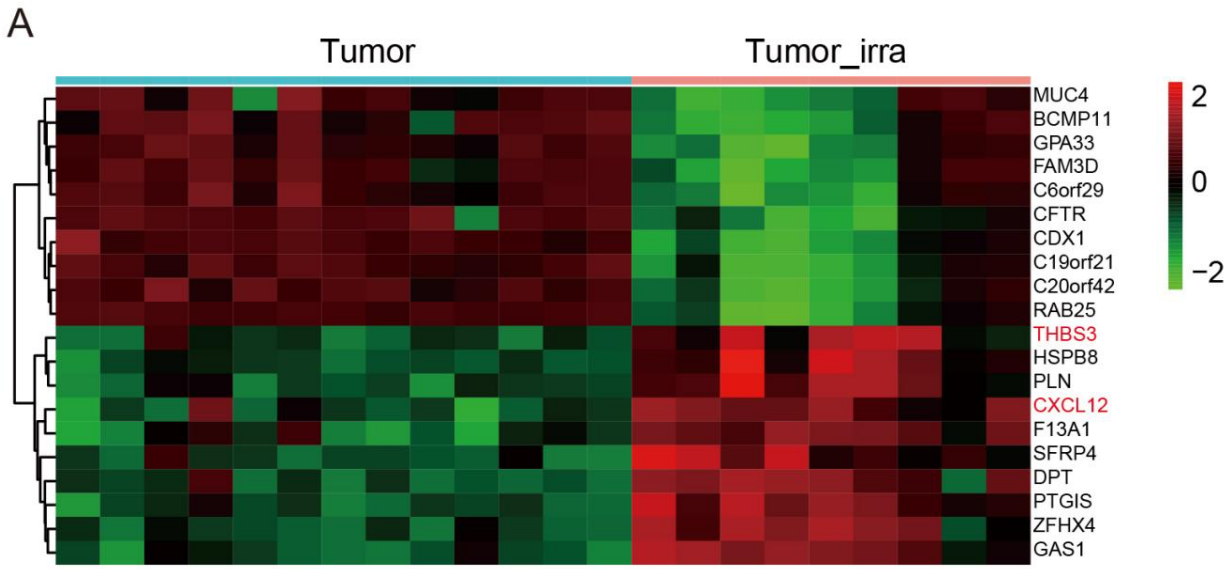

Fig. 1: Differential expressed genes in CRC tumor sample and tumor_irra sample; THBS3 and CXCL12 were high-expressed in Tumor_irra sample

THBS3 and *CXCL12* genes are two abnormally highly expressed genes in the CRC tumour irradiated group, which suggests that these two genes are related to the tolerance of CRC to radiotherapy and chemotherapy.

Signaling pathways detection in CRC

GESA was performed to analyze the functional and pathway enrichment of identified DEGs. KEGG analysis results showed the correlative signaling pathway in disease, such as cytokine-cytokine receptor interaction, JAK-STAT signalign pathway, PI3K/AKT signaling pathway and MAPK signaling pathway. The joyplot and dotplot were hired to visualize the enrichment pathways (Fig. 2A-2B). Take the intersection of joyplot and dotplot enriched pathways to draw a Venn diagram and the results showed that four pathways (cytokine-cytokine receptor interaction, JAK-STAT signalign pathway, PI3K/AKT signaling pathway and MAPK signaling pathway) were obtained (Fig. 2C).

The gene expression in cytokine-cytokine receptor interaction pathway

In the above four signal pathways related genes and screened out differential genes for intersection analysis and draw a Venn diagram. Except for the JAK STAT signaling pathway, more than 15% of the genes in the other three signaling pathways were differentially expressed. Therefore, the differential genes of these three signaling pathways were further screened. Use the pheatmap to perform differential analysis and GSEA analysis on the cytokine-cytokine receptor interaction, PI3K/AKT signaling pathway and MAPK signaling pathway. These three signal pathways that are significantly up-regulated in the tumor tissue after preoperative radiotherapy and we explored their activation and inhibition in genes expression level (Figs. 3, 4). In addition, cytokine-cytokine receptor interaction pathway is related to cell stemness, and we try to explore its relationship with the *CXCL12* gene.

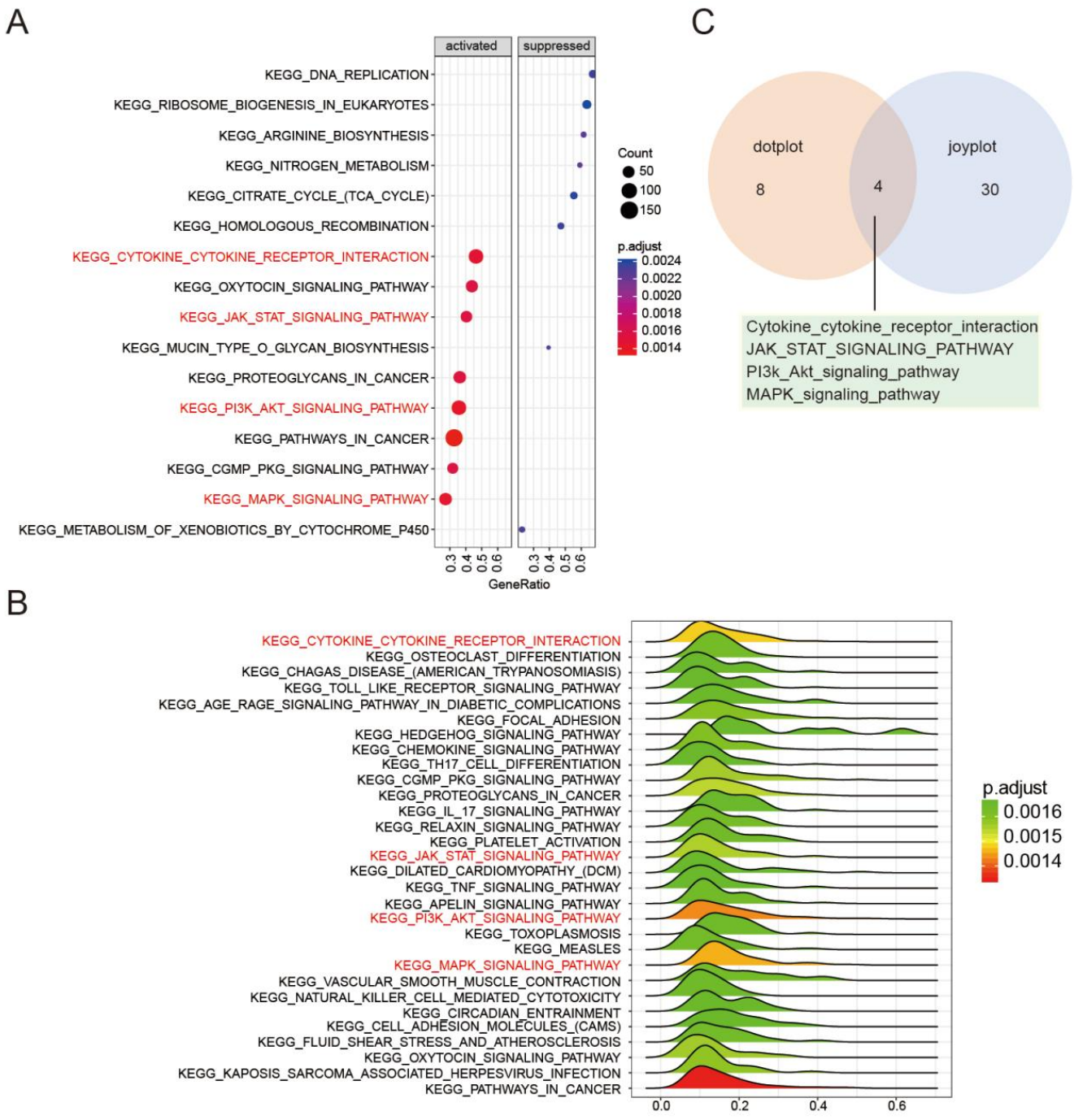

Fig. 2: Enrichment analysis of the most relevant signaling pathways by dotplot (A) and joyplot (B); The four common signaling pathways analysis by veen diagram were cytokine-cytokine receptor interaction, JAK-STAT signaling pathway, PI3K/AKT signaling pathway and MAPK signaling pathway (C)

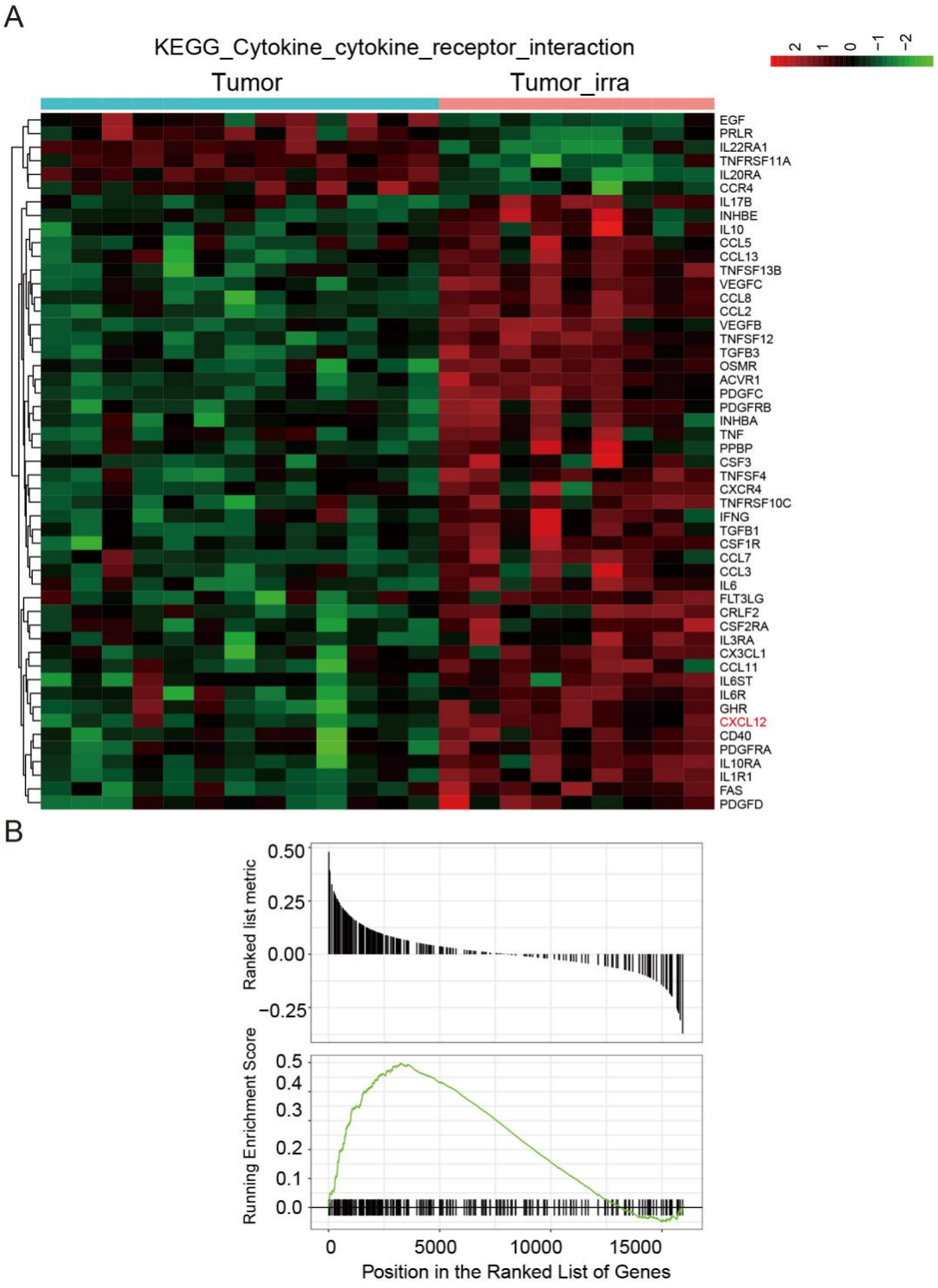

Fig. 3: CXCL12 was an important bio-maker in cytokine-cytokine receptor interaction signaling pathway by KEGG enrichment analysis

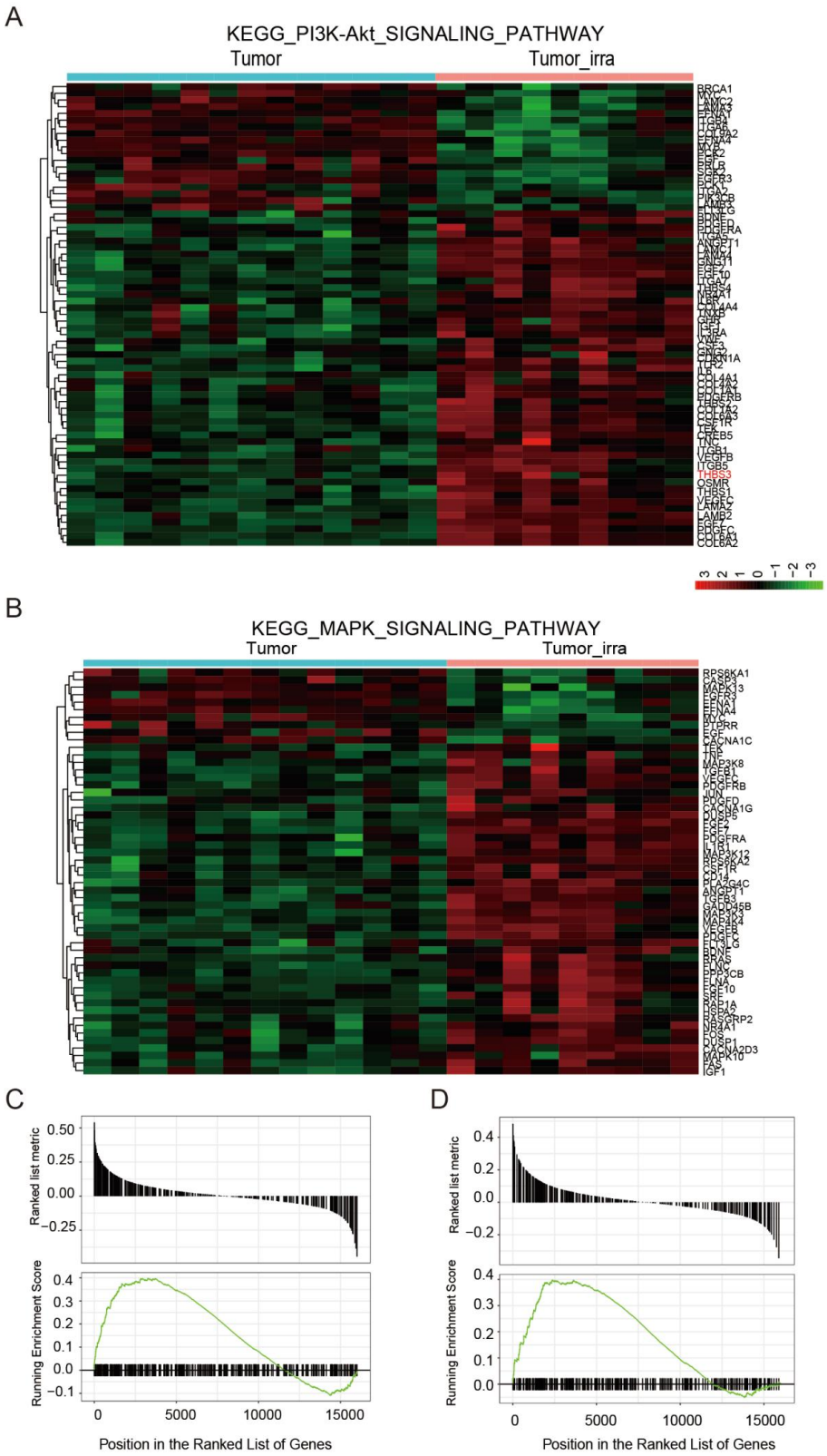

Fig. 4: KEGG enrichment analysis of PI3K/AKT signaling pathway (A&C) and MAPK signaling pathway (B&D)

The expression of CXCL12 gene in CRC

To verify the expression of *CXCL12* in CRC cells, quantitative real-time PCR was used and detected mRNA expression. The results showed that the expression of *CXCL12* in LoVo cell and SW480 cell was increased compared with FHC cell (Fig. 5A). Subsequently, the transfection efficiency of constructed *CXCL12* overexpression vector was detected and the results showed that the expression of *CXCL12* was up-regulated in LoVo cell and SW480 cell (Fig. 5B). In addition, the effect of overexpression of *CXCL12* on the proliferation ability of CRC cells was tested, and the results showed that *CXCL12* promoted the proliferation of LoVo cell and SW480 cell (Fig. 5C-5D).

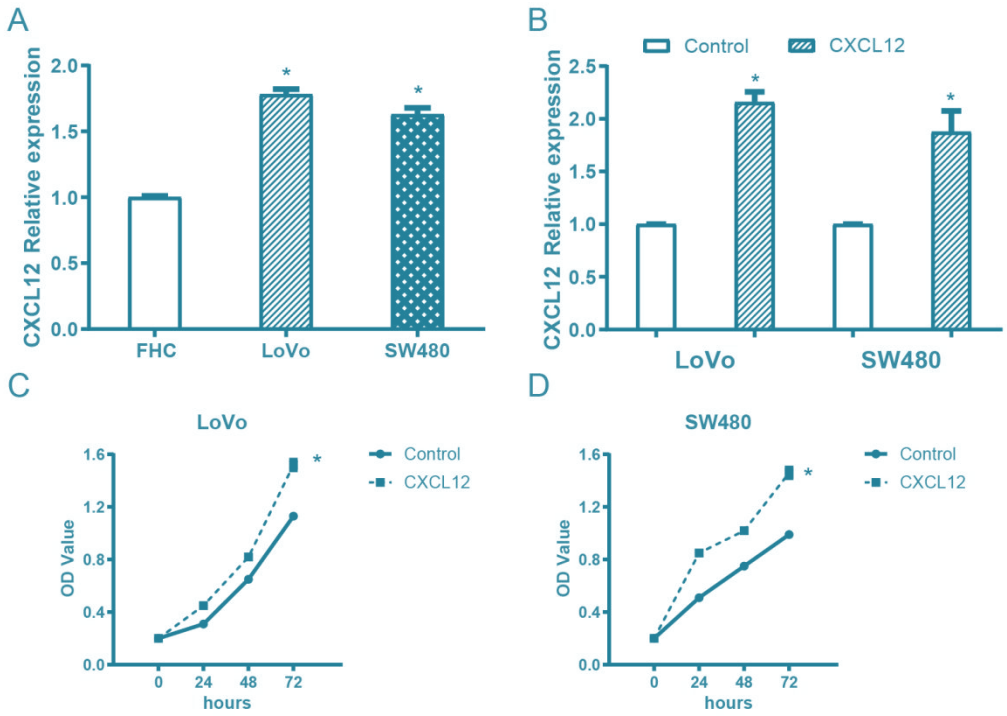

Fig. 5: The expression of CXCL12 in CRC cells was increased (A). The expression of CXCL12 was up-regulated in overexpressed-CXCL12 treatment group (B). The cell proliferation was promoted in overexpressed-CXCL12 treatment group both in LoVo cell (C) and SW480 cell (D)

Chemoresistance conferred by CXCL12 in CRC cells

Through LoVo cell and SW480 cell, the chemotherapy drug resistance situation was simulated *in vitro* by overexpression of *CXCL12* in CRC cells. The results were proved that CRC-derived cells (LoVo and SW480) were significantly more resistant to the anticancer drugs 5-FU at 48 h and 72 h after transfected with *CXCL12*, compared to the empty vector (control group) (Fig. 6A-6B).

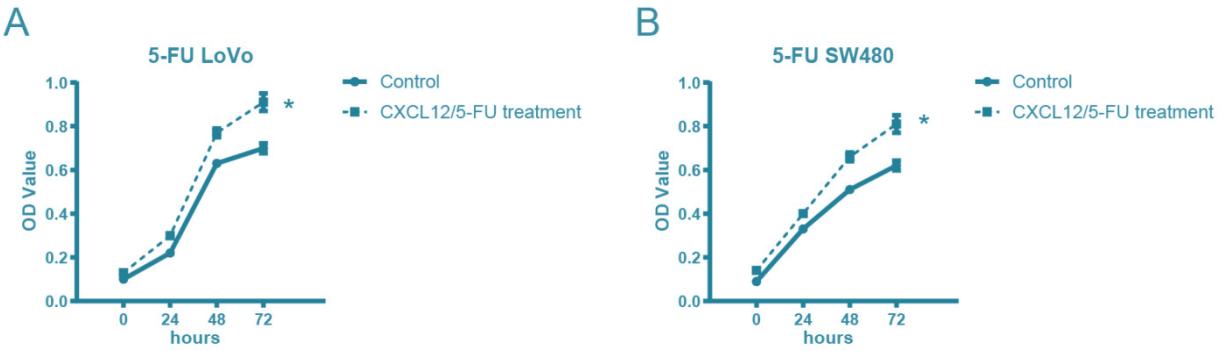

Fig. 6: Overexpression of CXCL12 increased the resistance of CRC cells to the anticancer drug 5-FU both in LoVo cell (A) and SW480 cell (B)

Sensitization of CRC cells to radiation by CXCL12 in CRC cells

To assess the radiation-enhancing effects after transfected with *CXCL12*, the cells were exposed to radiation treatment. Fig. 7A-7B displayed the survival curves in LoVo cell and SW480 cell for treatment with radiation. The results uncovered that there were significant differences concerning survival between two groups. The sensitizer enhancement ratio (SER) indicated that *CXCL12* increased the radiosensitivity of CRC cells to radiation. Moreover, compared to the empty vector (control group), LoVo and SW480 cells presented greater resistance to radiation due to upregulation *CXCL12* levels, and such results were detected both 48 and 72 hours after the cells were treated with radiation (Fig. 7C-7D).

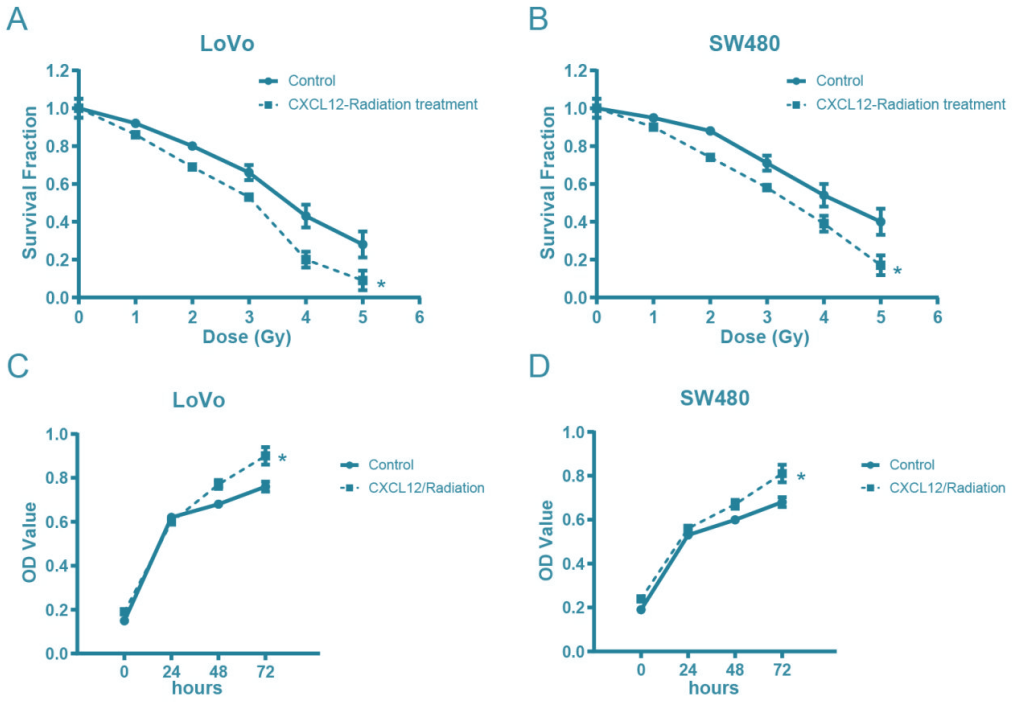

Fig. 7: The sensitizer enhancement ratio (SER) indicated that CXCL12 increased the radiosensitivity of LoVo cell (A) and SW480 cell (B) by survival curves. Overexpression of CXCL12 conferred stronger radioresistance in LoVo cells (C) and SW480 cells (D)

Discussion

CRC is amongst the leading causes of cancer related deaths worldwide (15). In this study, abnormal gene expression profiles in colorectal cancer were obtained by performing genome-wide microarray analysis, and *CXCL12* ranked among the top abnormally expressed genes. And the signal pathways closely related to *CXCL12* was obtained in colorectal cancer rely on the bioinformatics analysis of the associated pathways. In addition, through the in vitro cell model, *CXCL12* can promote the chemotherapy and radiotherapy tolerance in CRC cells under the treatment of chemotherapeutic drugs and radiotherapy. Since this is the standard of patient care, it is impossible to observe the effect of radiation on the tumor tissue. Obviously, our research is of great help to clinical studies.

Chemokine CXCL12 and its receptors CXCR4 and CXCR7 play an important role in regulation of homeostasis under normal physiological conditions (16, 17). CXCL12 is expressed constitutively in the most common sites for colon cancer metastasis, which reminds us that it is related to the malignancy degrees of CRC (18,19). The study found that *CXCL12* expression analysis can be used as a prognostic biomarker for cancer (20). This study confirmed the abnormally high expression of *CXCL12* through the DEGs profile analysis in CRC, and also found that the important correlation effect of *CXCL12* in a number of key signal pathways, including pathways related to cell stemness, cytokine-cytokine receptor interaction.

Currently, radiotherapy and chemotherapy are the main treatment methods for CRC (21,22). Preoperative external beam radiotherapy has been shown to increase pathological complete remission and reduce the probability of local recurrence (23) and also 5-Fluorouracil is the first line of chemotherapy in colorectal cancer (24). Regarding the effect of radiation-induced on cancer cells: It was confirmed that the expression of EGFR in cancer cells increased under the radiation-induced, and blocking the EGFR signal made the cells sensitive to the effects of radiation (25, 26). In addition, intra-cell-conversion of prodrug 5-FC to 5-fluorouracil was able to induce endocytosed exosome-trigger-dependent tumor cells death (27). Similarly, in this study, we used the CRC cell line to detect the chemosensitivity and radiotherapy tolerance with the participation of abnormally expressed gene *CXCL12*.

Conclusion

Our study efficiently identified several candidate targets that can potentially serve as biomarkers in the diagnosis of CRC. The functional signal pathways closely related to *CXCL12* have also been confirmed. Furthermore, we also demonstrated the influence of *CXCL12* in chemosensitivity and radiotherapy tolerance of CRC.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

No funding was received in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Malki A, ElRuz RA, Gupta I, Allouch A, Vranic S, Al Moustafa AE (2020). Molecular Mechanisms of Colon Cancer Progression and Metastasis: Recent Insights and Advancements. *Int J Mol Sci,* 22(1):130.
2. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wal-

lace MB (2019). Colorectal cancer. *Lancet,* 394(10207):1467-1480.
3. Mármol I, Sánchez-de-Diego C, Pradilla Dieste A, Cerrada E, Rodriguez Yoldi MJ (2017). Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. *Int J Mol Sci,* 18(1):197.
4. Kuipers EJ, Grady WM, Lieberman D, et al (2015). Colorectal cancer. *Nat Rev Dis Primers,* 1:15065.
5. Pajonk F, Vlashi E, McBride WH (2010). Radiation resistance of cancer stem cells: the 4 R's of radiobiology revisited. *Stem Cells,* 28(4):639-48.
6. Vega P, Valentín F, Cubiella J (2015). Colorectal cancer diagnosis: Pitfalls and opportunities. *World J Gastrointest Oncol,* 7(12):422-33.
7. Kreso A, Dick JE (2014). Evolution of the cancer stem cell model. *Cell Stem Cell,* 14(3):275-91.
8. Prasetyanti PR, Medema JP (2017). Intra-tumor heterogeneity from a cancer stem cell perspective. *Mol Cancer,* 16(1):41.
9. Meerson A, Khatib S, Mahajna J (2021). Natural Products Targeting Cancer Stem Cells for Augmenting Cancer Therapeutics. *Int J Mol Sci*, 22(23):13044.
10. Das PK, Zahan T, Abdur Rakib M, Khanam JA, Pillai S, Islam F (2019). Natural Compounds Targeting Cancer Stem Cells: A Promising Resource for Chemotherapy. *Anticancer Agents Med Chem,* 19(15):1796-1808.
11. De Angelis ML, Francescangeli F, Zeuner A, Baiocchi M (2021). Colorectal Cancer Stem Cells: An Overview of Evolving Methods and Concepts. *Cancers (Basel),* 13(23):5910.
12. Yamashita T, Ji J, Budhu A, et al (2009). EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology,* 136(3):1012-24.
13. Paolillo M, Colombo R, Serra M, et al (2019). Stem-Like Cancer Cells in a Dynamic 3D Culture System: A Model to Study Metastatic Cell Adhesion and Anti-Cancer Drugs. *Cells,* 2019, 8(11):1434.
14. Ren Y, Deng R, Cai R, et al (2020). TUSC3 induces drug resistance and cellular stemness via Hedgehog signaling pathway in colorectal cancer. *Carcinogenesis,* 41(12):1755-1766.
15. Rawla P, Sunkara T, Barsouk A (2019). Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. *Prz Gastroenterol,* 14(2):89-103.
16. Teicher BA, Fricker SP (2010). CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res,* 16(11):2927-31.
17. Miller MC, Mayo KH (2017). Chemokines from a Structural Perspective. *Int J Mol Sci,* 18(10):2088.
18. Sun X, Cheng G, Hao M, et al (2010). CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression. *Cancer Metastasis Rev,* 29(4):709-22.
19. Ray P, Stacer AC, Fenner J, et al (2015). CXCL12-γ in primary tumors drives breast cancer metastasis. *Oncogene,* 34(16):2043-51.
20. Samarendra H, Jones K, Petrinic T, et al (2017). A meta-analysis of CXCL12 expression for cancer prognosis. *Br J Cancer,* 117(1):124-135.
21. Hummel R, Hussey DJ, Haier J (2010). MicroRNAs: predictors and modifiers of chemo- and radiotherapy in different tumour types. *Eur J Cancer,* 46(2):298-311.
22. Cui SY, Huang JY, Chen YT, et al (2013). Let-7c governs the acquisition of chemo- or radioresistance and epithelial-to-mesenchymal transition phenotypes in docetaxel-resistant lung adenocarcinoma. *Mol Cancer Res,* 11(7):699-713.
23. Smith JA, Wild AT, Singhi A, et al (2012). Clinicopathologic Comparison of High-Dose-Rate Endorectal Brachytherapy versus Conventional Chemoradiotherapy in the Neoadjuvant Setting for Resectable Stages II and III Low Rectal Cancer. *Int J Surg Oncol,* 2012:406568.
24. Kishore C, Sundaram S, Karunagaran D (2019). Vitamin K3 (menadione) suppresses epithelial-mesenchymal-transition and Wnt signaling pathway in human colorectal cancer cells. *Chem Biol Interact,* 309:108725.
25. Kim K, Wu HG, Jeon SR (2015). Epidermal growth factor-induced cell death and radiosensitization in epidermal growth factor receptor-overexpressing cancer cell lines. *Anticancer Res,* 35(1):245-53.
26. Xiong Y, Shao L, Liu J, et al (2021). The Evolving Strategy of Californium-252 Neutron Intracavitary Brachytherapy in Treating Patients With Low-Lying T2 or T3 Rectal Adenocarcinoma: From Fixed to Individualized Regime With Intrarectal Peritumoral Injection of Amifostine. *Front Oncol,* 11:758698.
27. Altanerova U, Jakubechova J, Benejova K, et al (2019). Prodrug suicide gene therapy for cancer targeted intracellular by mesenchymal stem cell exosomes. *Int J Cancer,* 144(4):897-908.