Abnormal N-glycosylated CPXM1 promotes gastric cancer metastasis through ITGB2 / FAK pathway

Jun Du

Wannan Medical College

Mengxiang Zhu

Wannan Medical College

Wenwu Yan

Wannan Medical College

Jin Guo Wang (✉ shwangjg@sina.com)

The First Affiliated Hospital of Wannan Medical College: Yijishan Hospital of Wannan Medical College

<https://orcid.org/0000-0002-6100-6022>

Primary research

Keywords: gastric cancer, CPXM1, N-glycosylation, metastasis, molecular mechanism

Posted Date: June 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-613113/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: To explore the molecular mechanism of CPXM1 in gastric cancer (GC) metastasis.

Methods: Based on the difference in expression of CPXM1 mRNA in GC tissues and normal gastric mucosa tissues in public databases, rt-PCR, WB and IHC staining experiments were performed to verify the expression of CPXM1 in fresh GC tissues. Bioinformatics analysis predicts the possible signal pathways of co-expression genes in CPXM1 overexpression group. The malignant phenotypes of GC cells was verified by in vivo and in vitro experiments. Using tunicamycin to inhibit the N-glycosylation of CPXM1 and constructing mutations. The plasmid further validated the specific N-glycosylation sites that affect the malignant phenotype of gastric cancer cells.

Results: CPXM1 mRNA expression was significantly increased in GC tissues. The results of IHC staining and WB indicated that there was no difference in the expression of CPXM1 at the protein level. Knocking down CPXM1 expression in HGC-27 cells can significantly inhibit its migration and invasion ability. Increasing the expression of CPXM1 in MGC-803 and BGC-823 cell lines can significantly promote its migration and invasion capabilities. CPXM1 can also promote the adhesion of GC cells and change the cytoskeleton structure. The results of in-vivo experiments show that CPXM1 can promote the metastasis of GC cells in the abdominal cavity of nude mice. Bioinformatics analysis showed that the co-expressed genes of the CPXM1 high expression group were mainly enriched in cell adhesion function, and the experimental results verified that CPXM1 may promote the adhesion and metastasis of gastric cancer cells through the ITGB2/FAK signaling pathway. Through N-glycosylation site prediction and WB results, it was found that CPXM1 had abnormal N-glycosylation in GC tissues. Tunicamycin can reverse the ability of CPXM1 that promote the invasion of GC cells. After respectively mutating the N-glycosylation sites on CPXM1, it was found that N210 site is the key site for CPXM1 to promote the adhesion and invasion of GC cells.

Conclusion: CPXM1 is upgraded in GC. CPXM1 can promote the adhesion, migration and invasion of GC cells and change cytoskeleton structure. The N210 site on CPXM1 is a key site affecting the malignant phenotype of GC.

Introduction

GC is the fifth most commonly diagnosed cancer and the third most lethal cancer in the world [1], and one of the most lethal cancers in China. The 5-year survival rate of early gastric cancer patients is very high. Once metastasis occurs, it will indicate bad prognosis. The molecular mechanism of GC metastasis is very complex. The general process is that the cancer cells first infiltrate normal tissues from the primary focus. When the capillary and microvessel are violated, they need to remain active in the circulatory system, then stay in the distant organs, and finally the cancer cells exit the vascular wall to complete the metastasis. The metastasis and recurrence of GC were also affected by the clinical pathological factors such as the degree of lymphatic and vascular infiltration[3], tumor stage[4], tumor location[5], tumor

size[6], pathological classification[7] and differentiation degree[8]. The recurrence and metastasis of gastric cancer is one of the main causes of death of GC patients, so it is very important to explore the molecular mechanism of GC metastasis.

Carboxypeptidase X, M14 family member 1 (CPXM1) is one of the family members of carboxypeptidase, which is a glycoprotein secreted in N-glycosylation and can bind to collagen[9]. CPXM1 was expressed instantaneously in the early stage of osteoclast formation and may be involved in RANKL induced osteoclast production[10]. CPXM1 is a positive regulatory factor of fat formation downstream of FGF1/BAMBI. It may promote adipose tissue proliferation by affecting extracellular matrix remodeling[11]. However, CPXM1 can only be secreted outside the cell after glycosylation, and it can also be combined with collagen type III of extracellular matrix[12]. In the early stage of this study, CPXM1 mRNA was found to be highly expressed in tissues in TCGA and oncomine databases, and the clinical stage and infiltration degree of cpxm1 were predicted by using the bioinformatics analysis. However, the expression of CPXM1 in tumor cells and its related functions have not been reported in literature, so the relationship between overexpression and GC needs to be studied. Similarly, more and more bioinformatics analysis results show that CPXM1 plays a potential role in breast cancer[13], thyroid cancer[14], myelodysplastic syndrome[15], and head and neck squamous cell carcinoma[16].

Materials And Methods

Public Data Analysis

The gene expression quantification data and clinical data of gastric carcinoma patients were downloaded from the GDC data portal (<https://portal.gdc.cancer.gov/>). The expression difference of CPXM1 in normal gastric tissue and gastric cancer tissue was verified in the GEPIA database (<http://gepia.cancer-pku.cn>). NetNGlyc 1.0 was used to predict putative N-Glycosylation sites (<http://www.cbs.dtu.dk/services/NetNGlyc/>)

GC patients and collection samples

From May 2019 to December 2019, 20 cases of fresh gastric cancer tissues and corresponding adjacent tissues were collected from the First Affiliated Hospital, Yijishan Hospital of Wannan Medical College. The tissues were collected and transported in dry ice, then stored in liquid nitrogen for a long time. The inclusion criteria of patients were as follows: 1, gastric cancer confirmed by gastroscopy and pathology; 2, patients without other tumors; 3, patients without chemotherapy or radiotherapy. Informed consent was signed by all patients participating in the study. The study was approved by the ethics committee of the First Affiliated Hospital, Yijishan Hospital of Wannan Medical College.

GC tissues HIC and bioinformatics analysis

The detailed operation of this part is as described[17].

Cell lines

Five gastric cancer cell lines (SGC-7901, MGC-803, BGC-823, HGC-27, MKN-45) and GES-1 were purchased from Shanghai cell bank of the Chinese Academy of Sciences and cultured in our laboratory for long-term preservation. The cells were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Beyotime, China) at 37 °C in a humidified cell incubator containing 5% CO₂.

Plasmid transfection

The GC cells were cultured on the 6-well plates for 24 hours to make the density reach 80% confluence. Lipofectamine 3000 reagent (L3000015, Thermo Fisher, USA) was diluted in opti-MEM medium (31985062, Thermo Fisher, USA). The plasmid (GenePharma, China) was diluted with opti-MEM medium, and then P3000 reagent was added. The two reagents need to be well mixed. The diluted plasmids were added into each tube of diluted Lipofectamine 3000 reagent at a ratio of 1:1, and then incubated for 15 minutes. The mixed reagent was added into the cells which were transfected for 48 hours. Western blotting was used to detect protein expression to determine the transfection efficiency.

Lentivirus transfection and establishment of stable cell line

The GC cells were cultured on the 96-well plates for 24 hours to make the density reach 20% confluence. 10 µl lentivirus stock solution (GenePharma, China) was added to each well. After 48–72 hours infection, the infection efficiency was observed under fluorescence microscope. When the infection rate was 90%, the cells from 96 well plate were transferred to 24 well plate. At this time, the cells were cultured in 2 µg/ml puromycin medium (P8230, Solarbio, China) for 2 weeks until the fluorescence expression rate of the cells was stable at 90%. Western blotting was used to detect protein expression to determine the transfection efficiency.

Cell invasion and migration assay

For cell migration assay, 1×10^5 cells were plated in the upper chamber of 24-well transwell plates (8-µm pore polycarbonate membrane, Corning, USA) with 200 µl serum-free medium, while the lower chamber loaded 600 µl RPMI-1640 medium containing 20% FBS. For cell invasion assay, the upper chamber of 24-well transwell plates were previously coated with MixGel ECM (E0282, Sigma, USA). After invasion and migration for 24 h, the chamber was transferred to a new 24-well transwell plate, which the lower chamber loading 4% paraformaldehyde to fix the cells. After 30 min, chamber was washed softly 3 times with PBS and stained with crystal violet (0.1%, Beyotime, China) for 30 min. At last, the cells in 5 random areas were counted and photographed by high definition camera microscope. All the experiments were repeated three times.

Cell adhesion assay

In cell adhesion assay, 24 well plates were coated with the rat tail type I collagen (C8062, Solarbio, China), washed with PBS 2–3 times, and then placed at 37 °C overnight. The treated cell suspension (about 50000 cells) was planted on the coated culture plate and placed in the 37 °C cell incubator for 30

minutes and 1 hour respectively. The liquid was removed and washed with PBS for 1–2 times. First, the cells were fixed with 4% paraformaldehyde solution for 20 minutes, washed gently for 1–2 times, and then added crystal violet staining solution, dyed for 20 minutes, gently washed 1–2 times, and finally taken photos in 3 areas randomly selected under the microscope.

Animal experiments

The treated GC cells which were cultured on 10 cm culture plates in the logarithmic growth phase were digested, suspended in PBS. Then the cells were injected into the nude mice intraperitoneally at the standard of 1×10^5 cells/100 μ l per nude mice. After the injection, rubbing the abdomen of the nude mice to make the cells evenly to distribute in the abdominal cavity. All 6-week-old male nude mice were divided into 4 groups: NC group and CPXM1 overexpression group, shNC group and shCPXM1 knockdown group (5 mice in each group). Nude mice were sacrificed 6 weeks after operation, and the number of tumor nodules in the abdominal cavity and liver surface nodules were counted.

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRNzol Universal Reagent (TIANGEN, China) according to the manufacturer's instruction. Total RNA (1 μ g) was reverse-transcribed using FastKing RT Kit with gDNase (TIANGEN, China). The primers for GAPDH, CPXM1 were:

GAPDH-F: 5'-GTCAAGGCTGAGAACGGAA-3'

GAPDH-R: 5'-AAATGAGCCCCAGCCTTCTC-3'

CPXM1-F: 5'-GGCGATCTATATGATGGAGCCT-3'

CPXM1-R: 5'-CCTGTGTGATAACACCCGAGAA-3'.

qRT-PCR was performed using SuperReal PreMix Plus (TIANGEN, China) on QuantStudio 3 Real time PCR instrument (Thermo Fisher Scientific, USA). The reaction program was set to the following parameters: 95 °C for 15 min; 40 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s; one cycle of 95 °C for 15 s, 60 °C for 1 min.

Protein extraction and western blotting

50mg fresh tissue was cleaned with cold phosphate-buffered saline (PBS), cut into small pieces and lysed in 500 μ l radioimmunoprecipitation assay (RIPA) lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, EDTA; Beyotime, China) with phenylmethane sulfonyl fluoride (PMSF; Beyotime, China) by homogenizer. 6-well plates seeded cell were cleaned with cold PBS and lysed in 150 μ l RIPA lysis buffer with PMSF. Tissue or cell lysate were centrifuged at 12,000 rpm for 5 min at 4 °C and the total protein was collected in the supernatant. When the total protein of cell supernatant was extracted, the cells were firstly seeded in 10 cm dishes with complete media. Then, the

complete media was replaced by 10 ml RPMI-1640 without PBS and the cells cultured 48 h. Finally, the cell supernatant was collected into Amicon Ultra-15 10K device and centrifuged at 4000 g for 1 h at 4 °C. All extracted protein was measured the protein concentration by bicinchoninic acid (BCA) protein assay reagent kit (Beyotime, China) for subsequent western blotting. 15–20 µg protein sample loaded into per lane were separated by 8% SDS-PAGE (Beyotime, China). After electrophoresis for 1 h 30 min, the protein was transferred to nitrocellulose membranes (Pall, Mexico), which were subsequently blocked with 5% bovine serum albumin (BSA; Biofroxx, Germany) for 1 h at room temperature. The blocked nitrocellulose membranes were incubated in the primary antibodies at 1:1000 dilution at 4°C overnight and then incubated with secondary antibodies at 1:5000 dilution for 1 h at room temperature. After each antibodies incubation, the membranes were washed three times using triethanolamine buffered saline with 0.1% Tween-20 (TBST) for 15 min. Finally, the ECL buffer was used to detect bolts by the X-ray film. The following primary antibodies were used: anti-CPXM1 (GTX119190, 1:1000, GeneTex,), anti-GAPDH (AF7021, 1:5000, Affinity, China), anti-ITGB2 (AF6399, 1:1000, Beyotime, China), anti-FAK (D507U, 1:1000, CST, USA), anti-pFAK (D20B1, 1:1000, CST, USA), anti-Src (32G6, 1:1000, CST, USA), anti-pSrc (D49G4, 1:1000, CST, USA), anti-Erk (137F5, 1:1000, CST, USA), anti-pErk (197G2, 1:1000, CST, USA), anti-MMP2 (40994, 1:1000, CST, USA), anti-MMP9 (13667, 1:1000, CST, USA).

Phalloidin staining

The treated cells were cultured on the slide for 24 hours to make the density reach 50% confluence. The culture medium was aspirated and washed with PBS which was preheated at 37 °C for 1–2 times. We used 4% formaldehyde solution in PBS for cell fixation, and fixed for 10 minutes at room temperature. It should be noted that avoid the fixative containing methanol components, because methanol may destroy actin during the fixation process. At room temperature, the cells were washed 2–3 times with PBS for 10 minutes each time, permeabilized with 0.5% Triton X-100 solution for 5 minutes, and then washed 2–3 times with PBS for 10 minutes each time. The cells were covered 200 µl of the prepared rhodamine-labeled phalloidin working solution, and incubated for 30 minutes at room temperature in the dark. It should be noted that in order to reduce the background, 1% BSA can be added to the rhodamine-labeled phalloidin working solution. In order to avoid volatilization of the solution during the incubation, the coverslip can be transferred to a sealed container. After the incubation, the slides were washed with PBS 3 times for 5 minutes each time. We Used 200µl of anti-fluorescence quencher (including DAPI) solution to counterstain the cell nucleus for about 30 seconds, washed the slides with PBS, and then placed it upside down on the coverslips which had been dripped with a drop of water-soluble mounting tablets. The excess mounting tablets was removed. The fluorescence microscope or confocal microscope, selected TRITC excitation/emission filter (Ex/Em = 540/570nm) and DAPI excitation/emission filter (Ex/Em = 364/454nm), was used to take pictures which selected randomly 3 areas.

Statistical analysis

Each experiments were repeated three times, and the results were analyzed by Student's t-test. All *p* values < 0.05 were considered to be statistically significant. R 4.0.2, Graphpad Prism 8.0.2, SPSS 22, Image J and other softwares were used for data processing, analysis and mapping.

Results

CPXM1 mRNA expression in GC is elevated

Firstly, we used the online GEPIA database to determine the expression level of CPXM1 mRNA in GC tissues. We found that CPXM1 mRNA was highly expressed in GC tissues which was compared with normal gastric mucosa tissues. (Fig.1A) To further confirm this result, we detected the expression of CPXM1 mRNA in 20 human GC tissues and matched normal gastric mucosa tissues by rt-PCR. As expected, the data showed that the expression level of CPXM1 mRNA in GC tissue was significantly increased (Fig.1B-C).

Abnormal glycosylation of CPXM1 protein in GC

Subsequently, we detected the protein expression of CPXM1 in 96 GC tissues and 84 normal gastric mucosa tissues by IHC. We found that there was no significant difference in the protein expression level of CPXM1 (Fig. 2A-B). However, we found that the expression of 120kDa CPXM1 protein in GC tissues was significantly higher than that in normal gastric mucosa tissues (Fig.2C). In order to verify whether this phenomenon was caused by N-glycosylation on CPXM1, we predicted the N-glycosylation sites on CPXM1 through online database. The results showed that there might be four glycosylation sites on CPXM1, which were N57, N210, N318 and N472 (Fig.2D).

CPXM1 knockdown inhibited cell migration and invasion in HGC-27 cells

To verify the expression level of CPXM1 in GC cell lines HGC-27, SGC-7901, MGC-803, BGC-823 and MKN-45 and normal gastric mucosa cell line GES-1, we found that the expression level of CPXM1 was the highest in HGC-27 and SGC-7901 cell lines, and the lowest in MGC-803 and BGC-823 cell lines (Fig.3A). We chose HGC-27, BGC-823 and MGC-803 cell lines for follow-up experiments. We chose HGC-27 cell line to knockdown the expression of CPXM1 to verify the effect of CPXM1 on the migration and invasion of GC cells. shNC as control group, shCPXM1-1, shCPXM1-2, shCPXM1-3 and shCPXM1-4 as experimental groups were used to observe the knockdown efficiency of CPXM1. The results showed that shCPXM1-1 and shCPXM1-2 had the most significant knockdown efficiency (Fig.3B). shCPXM1-1 and shCPXM1-2 were selected to package the virus to establish HGC-27 CPXM1 knockdown stable transfection strain. The results showed that the migration and invasion ability of GC cells decreased significantly after CPXM1 knockdown ($p < 0.001$) (Fig.3C).

CPXM1 overexpression promoted cell migration and invasion in BGC-823 cells and MGC-803 cells

BGC-823 and MGC-803 were selected for CPXM1 plasmid transient transfection and lentivirus stable transfection strains for in vivo experiments. The transiently transfected BGC-823 GC cell line was verified by WB to confirm that it expressed CPXM1 protein (Fig.4A). We tested its migration and invasion ability and found that the migration and invasion ability of the BGC-823 cell line was significantly improved after transient expression of CPXM1 (Fig.4C). Stably transfected MGC-803 GC cell line was verified by WB to confirm that it expressed CPXM1 protein (Fig.4B). Simultaneously testing its migration and metastasis

ability, it was found that the migration and invasion ability of MGC-803 cell line was significantly improved after CPXM1 was stably expressed (Fig.4D). In addition, the cytoskeleton arrangement and pseudopodia of the MGC-803 cells stably transfected with CPXM1 were also significantly changed (Fig.4E).

CPXM1 promoted the metastasis of GC cells in vivo

In order to verify whether CPXM1 affects the metastatic ability of GC cells in vivo, stable CPXM1 overexpression cell lines and stable CPXM1 knockdown cells were injected into the abdominal cavity of nude mice. The nude mice were raised in SPF IVC cage for 4 weeks. Then the nude mice were killed, and the number of intraperitoneal cancer nodules was calculated. The results showed that overexpression of CPXM1 could significantly increase the number of abdominal metastases in nude mice (the number of intestinal and liver metastases increased significantly) (FigA-C). Reversal of CPXM1 expression could significantly inhibit the abdominal metastases in nude mice (the number of intestinal and liver metastases increased significantly) (FigD-F).

CPXM1 promoted GC metastasis through N-glycosylation

In order to verify that CPXM1 is a glycosylated protein and its glycosylation affects the metastasis of GC, we used 10 µg/ml tunicamycin which could block the formation of N-glycosidic bond by inhibiting the transfer of N-acetylglucosamine-1-phosphate to polyterpenoid monophosphate) for 24 hours. The results showed that the molecular weight of CPXM1 decreased after tunicamycin treatment (Fig.6A), and tunicamycin could reverse the ability of CPXM1 overexpression to promote GC invasion (Fig.6B-C).

N210 was the key site of CPXM1 promoting GC metastasis

We found that CPXM1 could contain four glycosylation sites (N57, N210, N318 and N472) through the N-glycosylation prediction website, in order to research which site affects the effect of CPXM1 on GC metastasis. These sites (N57Q, N210Q, N318Q and N472Q) were mutated respectively (Fig.7A), and then transiently transfected into MGC-803 cells. The results showed that although the molecular weight of each mutation group was reduced to different degrees (Fig.7B), only N210Q could significantly reduce the migration (Fig7.C), invasion (Fig7.D) and adhesion (Fig7.E-F) of GC.

CPXM1 promoted GC metastasis through ITGB2 / FAK signaling pathway

We found that CPXM1 could enrich in cell adhesion signaling pathway (Fig.8A-B), and CPXM1 was significantly correlated with the expression of ITGB2, MMP2 and MMP9 (Fig.8C). We examined the adhesion related signaling pathways in CPXM1 knockdown group, overexpression group and N210Q mutation group, and found that CPXM1 could significantly reduce the expression of p-FAK, p-Src, p-Erk, MMP2 and MMP9.

Discussion

After screening a large number of differentially expressed genes in the public database, our team found that the expression of CPXM1 mRNA in GC tissues was significantly higher than that in normal gastric mucosa tissues. At the same time, literature review found that CPXM1 has not been studied in cancer. So we speculate that CPXM1 may play a key role in the occurrence and development of GC. Subsequently, rt-PCR confirmed that the expression of CPXM1 mRNA was increased in 20 cases of GC. However, the expression of CPXM1 in GC tissues and normal gastric mucosa tissues was not statistically significant. This result is quite different from that of rt-PCR. CPXM1 was predicted to be an N-glycosylated protein by literature review and website prediction. WB results showed that there were several bands of CPXM1 in GC, which were located at 80,100,120kDa. However, in normal gastric mucosa, CPXM1 was located at 80,100 kDa. CPXM1 has been proved to be a glycoprotein secreted by N-glycosylation, and only CPXM1 at 120 kDa can be secreted out of cells[9]. Therefore, it may be that GC cells secrete CPXM1 protein, which leads to the inconsistent expression of CPXM1 mRNA and CPXM1 protein in GC cells. However, IHC staining showed that the survival rate of patients with high expression of CPXM1 was significantly lower than that of patients with low expression of CPXM1. There was no significant difference in overall survival between the high and low expression groups of CPXM1 in normal gastric mucosa. This suggests that only the expression of CPXM1 in GC can predict the overall survival of GC patients. Similarly, the expression of CPXM1 was significantly correlated with tumor size and lymph node metastasis grade. Univariate and multivariate analysis showed that CPXM1 could be used as an independent prognostic factor for GC. CPXM1 may have similar functions to its homologous family members CPXM2 and ACLP, which may play an important role in tumor progression[18–19]. Since CPXM1 is only studied in adipocytes and osteoclasts[10–11], the molecular mechanism of CPXM1 in tumor still needs to be verified.

Through bioinformatics analysis, our team found that the co-expression genes of CPXM1 high expression group were mainly enriched in cancer-related pathways, among which the adhesion function was the most significant. This suggests that CPXM1 may play a key role in the occurrence and development of GC. After screening GC cell lines, we found that CPXM1 was highly expressed in HGC-27 cell lines, but hardly expressed in BGC-823 and MGC-803 cell lines. Therefore, we used HGC-27 stable CPXM1 knockout strain, MGC-803 stable CPXM1 expression strain and BGC-803 transient CPXM1 expression strain to do in vitro functional experiments. The results showed that the migration and invasion ability of HGC-27 decreased significantly after CPXM1 knockdown. On the contrary, the migration and invasion ability of BGC-823 and MGC-803 cell lines were significantly improved after CPXM1 overexpression. At the same time, according to the results of bioinformatics analysis, CPXM1 can be associated with adhesion and cytoskeleton changes. The results showed that CPXM1 could significantly promote the adhesion of GC cells, change the cytoskeleton structure, and promote the formation of foot process of GC cells. The results showed that CPXM1 promoted the intraperitoneal metastasis of GC cells in nude mice, and the inhibition of CPXM1 expression in stable cell line could inhibit the metastasis of GC cells. These results suggest that CPXM1 can promote the malignant phenotype of GC cells. We found that CPXM1 may

regulate integrin protein ITGB2 through FAK/Src/Erk to change the expression of MMP2 and MMP9, and affect the metastasis of GC cells.

In order to explore the effect of N-glycosylation of CPXM1 on GC cells, we used tunicamycin to treat overexpression cell lines, and observed whether CPXM1 affected a series of malignant phenotypes of GC cells by inhibiting the glycosylation of CPXM1. The invasion ability of GC cells was significantly different between DMSO-NC group and DMSO-CPXM1 group, which was consistent with the results of previous overexpression group. There was no significant difference between DMSO-NC group and Tuni-NC group in the invasion ability of GC cells, indicating that the dose of 10 μ g/ml tunicamycin did not affect the invasive ability of GC cells. There was a significant difference between CPXM1-DMSO group and CPXM1-tuni group in the invasion ability of GC cells, indicating that inhibition of N-glycosylation level of CPXM1 can reverse the enhanced invasion ability of GC cells induced by CPXM1. Therefore, N-glycosylation of CPXM1 can promote the invasion and metastasis of GC cells. There are four glycosylation sites in CPXM1: N58, N210, N318 and N472. In order to further explore which site can affect CPXM1 to promote GC cell metastasis, we mutated these sites to prevent normal glycosylation. We set up NC group, CPXM1 group, N58Q group, N210Q group, N318Q group and N472Q group for adhesion and invasion experiments. After transfection, WB test was carried out in each group, and it was found that the molecular weight of each group had a slight change. This may be due to the small molecular weight of single site glycosyl. However, the molecular weight of N58Q and N210Q changed the most. The results showed that mutant N210 glycosylation site can significantly reduce the adhesion and invasion of GC cells, and inhibit ITGB2/FAK signaling pathway. In conclusion, N210 on CPXM1 is the key site of CPXM1 promoting the migration and invasion of GC cells.

In this study, we screened related genes through public databases, analyzed the possible mechanism of CPXM1 in GC by bioinformatics, and further verified its molecular mechanism in GC metastasis through in vivo and in vitro experiments. In the experiment, we found that the expression of CPXM1 mRNA and protein was inconsistent. By consulting the literature and websites, we predicted the abnormal N-glycosylation of GC cells, and by inhibiting the N-glycosylation of CPXM1 and mutant glycosylation sites, we found the effect of N210 site on the migration and invasion of GC cells. The effect of glycosylation sites on protein folding. Because N210 is located in the discoid domain of CPXM1, DSD is known to bind to collagen[20]. Whether the mutated N-glycosylation site will change the structure of DSD and affect its binding with collagen still needs further verification.

However, there are still some deficiencies in this experiment, which need to be supplemented by the follow-up experiments. First, the previous literature results showed that CPXM1 could be secreted out of the cell only at 120 kDa, and WB results showed that the content of 120 kDa in GC tissue was higher. Therefore, we speculate that the expression of CPXM1 protein is not uniform due to the secretion of CPXM1 protein by GC cells. This conjecture needs to be further verified by measuring the content of CPXM1 in serum between patients with GC and normal people. Secondly, through bioinformatics analysis and WB verification, we found that GC may regulate the expression of integrin protein ITGB2, change the expression of MMP2 and MMP9 functional proteins through FAK/Src/Erk, and affect the metastasis of

GC cells. However, this result is only speculation, and further verification of pathway proteins and phenotypes of GC cells is needed by FAK inhibitor or shrna-itgb2. Thirdly, the references are also obtained through website prediction. This study also assumes that there are only four N-glycosylation sites on CPXM1. This accurate result can only be obtained by mass spectrometry, but the laboratory is lack of relevant instruments. However, these deficiencies need to be verified by subsequent experiments, and these deficiencies are not enough to affect the novelty of experimental content, the accuracy of experimental methods, the clarity of experimental ideas and the effectiveness of experimental results. The results provide a new idea for the diagnosis and treatment of GC.

Conclusion

The results showed that the expression of CPXM1 was increased in GC. Overexpression of CPXM1 could promote the adhesion, invasion and migration of GC. N210 glycosylation on CPXM1 is a key site in promoting GC metastasis through ITGB2/FAK/Src/Erk/MMP2/MMP9 pathway.

Declarations

Ethics approval and consent to participate

This study was approved by the Scientific Research and New Technology of Wanan Medical College Yijishan Hospital IRB, and the ethics approval number is 202008. All patients signed the informed consent before surgery.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

DJ designed the study and wrote the manuscript. YWW extracted the data from various public databases and collected GC samples. DJ and ZXM processed the data through R and Perl software and carried out

various experimental operations. WJG resolved any disputes, provided critical evaluation and supervised the study. All authors contributed to this article and agreed to publish the final manuscript.

Competing interests

The authors declare that they have no conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, *et al.* *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* *CA: A Cancer Journal for Clinicians*, 2018, 68(6):394–424.
2. Chen W, Zheng R, Baade P D, *et al.* *Cancer statistics in China, 2015.* *CA: A Cancer Journal for Clinicians*, 2016, 66(2):115–132.
3. Saito H, Murakami Y, Miyatani K, *et al.* Predictive factors for recurrence in T2N0 and T3N0 gastric cancer patients. *Langenbeck Archives of Surgery*, 2016, 401(6):823–8.
4. Fukuchi M, Ishiguro T, Ogata K, *et al.* Risk Factors for Recurrence After Curative Conversion Surgery for Unresectable Gastric Cancer. *Anticancer research*, 2015, 35(11):6183–8.
5. Lee JY, Cho K B, Kim E S, *et al.* Risk factors for local recurrence after en bloc endoscopic submucosal dissection for early gastric cancer. *World Journal of Gastrointestinal Endoscopy*, 2016, 8(7):330.
6. Jin LX, Moses L E, Squires M H, *et al.* Factors Associated With Recurrence and Survival in Lymph Node–negative Gastric Adenocarcinoma. *Ann Surg*, 2015, 262(6):999.
7. Kang WM, Meng Q B, Yu J C, *et al.* Factors associated with early recurrence after curative surgery for gastric cancer. *World J Gastroenterol*, 2015, 21(19):5934–40.
8. Cao L, Selby L V, Hu X, *et al.* Risk factors for recurrence in T1-2N0 gastric cancer in the United States and China. *J Surg Oncol*, 2016, 113(7):745–9.
9. Lei Y, Xin X, Morgan D, *et al.* Identification of Mouse CPX-1, a Novel Member of the Metalloprotease Gene Family with Highest Similarity to CPX-2. *DNA Cell Biol*, 1999, 18(2):175–85.
10. Chang EJ, Kwak H B, Kim H, *et al.* Elucidation of CPX-1 involvement in RANKL-induced osteoclastogenesis by a proteomics approach. *FEBS Lett*, 2004, 564(1–2):0–170.
11. Kim YH, Barclay J L, He J, *et al.* *Identification of carboxypeptidase X (CPX)-1 as a positive regulator of adipogenesis.* *Faseb Journal*, 2016, 30(7).
12. Kim YH, O'Neill H M, Whitehead J P. Carboxypeptidase X-1 (CPX-1) is a secreted collagen-binding glycoprotein. *Biochemical Biophysical Research Communications*, 2015, 468(4):894–9.
13. Mao XH, Ye Q, Zhang GB, *et al.* Identification of differentially methylated genes as diagnostic and prognostic biomarkers of breast cancer. *World J Surg Oncol*, 2021, 19(1):29.
14. Kumar A, Bandapalli OR, Paramasivam N, *et al.* Familial Cancer Variant Prioritization Pipeline version 2 (FCVPPv2) applied to a papillary thyroid cancer family. *Scientific reports*, 2018, 8(1):11635.

15. Wang YH. Lin CC, Yao CY, et al A 4-gene leukemic stem cell score can independently predict the prognosis of myelodysplastic syndrome patients. *Blood advances*, 2020, 4(4):644–54.
16. Chen Y. Li ZY, Zhou GQ. Sun Y. An Immune-Related Gene Prognostic Index for Head and Neck Squamous Cell Carcinoma. *Clinical cancer research*, 2021, 27(1):330–41.
17. Du J, Zhu M, Yan W, et al. CPXM1: a novel biomarker for gastric cancer prognosis, 22 March 2021, PREPRINT (Version 1) available at Research Square [<https://doi.org/10.21203/rs.3.rs-337128/v1>].
18. Niu G. Yang Y, Ren J, et al Overexpression of CPXM2 predicts an unfavorable prognosis and promotes the proliferation and migration of gastric cancer .*Oncology Reports*, 2019 Oct;42(4):1283–1294.
19. Majdalawieh AF, Massri M, Ro HS. AEBP1 is a Novel Oncogene: Mechanisms of Action and Signaling Pathways. *Journal of Oncology*, 2020 May 27;2020:8097872.
20. Schissel SL. Dunsmore SE, Liu X, *et al*. Aortic Carboxypeptidase-Like Protein Is Expressed in Fibrotic Human Lung and its Absence Protects against Bleomycin-Induced Lung Fibrosis. *Am J Pathol*, 2009, 174(3):0–828.

Figures

Figure 1

CPXM1 mRNA expression in GC is elevated. A.The expression of CPXM1 mRNA in GC tissues and normal gastric mucosa tissues in GEPIA database. The results showed that the expression of CPXM1 mRNA in GC tissues was significantly upgraded ($p < 0.001$). B-C. Twenty fresh GC samples were collected. RT-PCR results showed that the average expression multiple of CPXM1 was 9.1, 1.25, 1.85, 4.5, 6.4, 0.45, 0.09, 9.75, 7, 8.1, 5.15, 4.15, 3.3, 5.5, 1.35, 0.95, 4.75, 4.35, 2.7 and 5.75 times. The dotted line which is 1 represents the relative expression multiple of CPXM1 mRNA in normal gastric mucosa tissues of each groups. Student's t-test showed that the expression level of CPXM1 mRNA in GC tissues was significantly higher than that in normal gastric mucosa tissues ($p < 0.001$). *** $p < 0.001$.

Figure 2

Abnormal glycosylation of CPXM1 protein in GC. A. IHC staining of GC tissues and normal gastric mucosa tissues. The left picture showed the expression of CPXM11 protein in GC tissues. The right picture showed the expression of CPXM1 protein in normal gastric mucosa tissues. B. The results of IHC staining in 96 gastric cancer tissues and 84 normal gastric mucosa tissues showed that the IHC scores of the two groups were not statistically significant ($p > 0.05$). C. After WB detection of the previous 20 pairs of GC samples, it was found that CPXM1 in GC tissues was highly glycosylated (most of them were at 100kDa or 120kDa position, a small number were at 80kDa position), and most of CPXM1 in normal gastric mucosa tissues were at 80kDa or 100kDa position. D. By predicting the N-glycosylation site of CPXM1 through online database, we found that there may be four N-glycosylation sites on CPXM1, which are located in N57, N210, N318, N472.

Figure 3

CPXM1 knockdown inhibited cell migration and invasion in HGC-27 cells. A. The expression of CPXM1 in normal gastric mucosa cell lines (GES-1) and five GC cell lines (HGC-27, MGC-803, BGC-823, MKN-45, SGC-7901). B. shCPXM1-1 group and shCPXM1-2 group were found to have the highest knockdown efficiency. C. The left figure showed the detection results of cell migration ability of HGC-27 transfected with NC lentivirus, shCPXM1-1 lentivirus and shCPXM1-2 lentivirus respectively. The right figure showed

that knockdown of CPXM1 reduced the relative migration ability based on Student's t-test ($p < 0.001$). D. The left figure showed the detection results of cell invasion ability of HGC-27 transfected with NC lentivirus, shCPXM1-1 lentivirus and shCPXM1-2 lentivirus respectively. The right figure showed that knockdown of CPXM1 reduced the relative invasion ability based on Student's t-test ($p < 0.001$). *** $p < 0.001$.

Figure 4

CPXM1 overexpression promoted cell migration and invasion in BGC-823 cells and MGC-803 cells. A. Results of transient transfection of NC plasmid and CPXM1 full-length plasmid into BGC-823 cell line. B. Results of stable transfection of NC lentivirus and CPXM1 lentivirus into MGC-803 cell line. C. The left figure showed the detection results of cell migration ability of the treated BGC-823 cell line. The right figure showed that overexpression of CPXM1 promoted the relative migration ability based on Student's t-test ($p < 0.001$). D. The left figure showed the detection results of cell invasion ability of the treated BGC-823 cell line. The right figure showed that overexpression of CPXM1 promoted the relative invasion ability based on Student's t-test ($p < 0.001$). E-F. The relative migration ($p < 0.01$) and invasion ($p < 0.001$) ability of the treated MGC-803 cell line were significantly improved. G. Compared with the NC control group, CPXM1 stable overexpressed MGC-803 was found to have disordered cytoskeleton and many pseudopodia after phalloidin staining. ** $p < 0.01$; *** $p < 0.001$.

indeed liver metastasis (right figure). D. The results of the cells injected into the abdominal cavity of nude mice in the shNC group and shCPXM1 knockdown group. E. The number of abdominal nodules in shCPXM1 group was significantly lower than that of the shNC group (left figure). No cancer nodules were found on the liver surface in shCPXM1 group, while liver metastasis was found in shNC group (right figure). The difference was statistically significant ($p < 0.001$). F. HE staining was made to verify that the nodule was cancer nodules (left and right figure), and confirmed that there was liver metastasis (middle figure). ** $p < 0.01$; *** $p < 0.001$. There were five nude mice in each groups.

Figure 6

CPXM1 promoted GC metastasis through N-glycosylation. A. The results of CPXM1 stable overexpressed MGC-803 treated with 10μg/ml tunicamycin for 24 hours showed that CPXM1 migrated from 120 kDa to 100 kDa position. B. The left figure showed the detection results of cell relative migration ability of the DMSO-NC, Tuni-NC, DMSO-CPXM1, Tuni-CPXM1 groups. The right figure, based on Student’s t-test, showed that overexpression of CPXM1 promoted the relative migration ability ($p < 0.001$) and

tunicamycin can reverse the ability of CPXM1 to promote the invasion of GC cells ($p < 0.001$). N.S. no significance. *** $p < 0.001$.

Figure 7

N210 was the key site of CPXM1 promoting GC metastasis. A. The mutated N-glycosylation sites were N57Q, N210Q, N318Q and N472Q, respectively. B.WB results showed that the molecular weight of the four mutant sites decreased in varying degrees. C-D. The relative migration ($p < 0.01$) and invasion ($p < 0.001$) ability of the CPXM1 N210 site mutation MGC-803 cells were significantly inhibited. E-F. The 30min adhesion ($p < 0.001$) and 60min adhesion ($p < 0.001$) ability of the CPXM1 N210 site mutation MGC-803 cells were significantly inhibited. * compared with NC group; # compared with CPXM1 group; **/## $p < 0.01$; ***/### $p < 0.001$.

Figure 8

CPXM1 promoted GC metastasis through ITGB2 / FAK signaling pathway. A. KEGG enrichment showed that CPXM1 might be involved in cell adhesion. B. CPXM1 may be involved in adhesion related signaling pathway. C. Correlation between CPXM1 and ITGB2, MMP2 and MMP9 co-expression. D-E. CPXM1 may promote gastric cancer metastasis through ITGB2/FAK signaling pathway. F. The N210 site on CPXM1 is a key site to promote gastric cancer metastasis through ITGB2/FAK signaling pathway.