Lumican promotes proliferation, migration and invasion of gastric cancer through ERK pathway

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Research Article

Keywords: gastric cancer, lumican, proliferation, migration, invasion, ERK pathway

Posted Date: March 14th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2679516/v1

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Abstract

Purpose
To clarify the molecular mechanism of lumican’s effects on gastric cancer cell proliferation, migration, and invasion.

Methods
qRT-PCR was used to analyze lumican expression in gastric cancer tissues and cell lines. Small interfering RNA (siRNA) transfection and lentivirus infection have been used to produce lumican knockdown or overexpression gastric cancer cell models from screened cell lines. CCK-8, wound healing assays, and transwell assays were performed to confirm the effect of lumican on gastric cancer cell’s proliferation, migration, and invasion. To further evaluate the potential mechanism of lumican on gastric cancer cells, bioinformatic prediction and western blot experiment were used to identify and confirm its related signaling pathway.

Results
Using MGC-803 and AGS gastric cancer cells, lumican knockdown or overexpression was achieved. Overexpression of lumican increased MGC-803 and AGS gastric cancer cell proliferation, migration, and invasion, whereas knockdown decreased them. The expression levels of ERK and p-ERK, two key proteins of the ERK pathway, were significantly decreased in MGC-803 and AGS cells with lumican knockdown, while the opposite result was observed with lumican overexpression; the expression levels of MEK and p-MEK, two key proteins of the MEK pathway, were not significantly changed with lumican knockdown or overexpression. GDC-0994, an ERK pathway inhibitor, restored ERK1/2 and p-ERK1/2 protein expression in MGC-803 and AGS cells overexpressing lumican.

Conclusion
Lumican was discovered in high levels in the tissues of patients with gastric cancer, and it promoted proliferation, migration, and invasion in gastric cancer cells. Lumican may impact the expression of two main proteins, ERK1/2 and p-ERK1/2, in the ERK signaling pathway rather than activating it via MEK, indicating that the pathway may be a therapeutic target for lumican overexpression in gastric cancer.

1. Background
The most recent study from the World Health Organization indicates that gastric cancer (GC) has the fifth highest incidence and the fourth highest fatality rate globally [1]. The majority of patients with GC are identified at an advanced stage[2], and the age-standardized five-year survival rate for GC was less than
30% in the majority of countries[3]. Although there are now no effective strategies to prevent the recurrence and spread of gastric cancer, it is vital to investigate the processes of gastric carcinogenesis and development in order to identify simple and efficient therapy targets for GC.

Lumican is a small leucine-rich proteoglycan that belongs to class II of small leucine-rich proteoglycans (SLRPs) [4]. Lumican coordinates signaling for cancer progression, promoting or inhibiting tumor cell proliferation, migration, or invasion with a positive or negative correlation to tumor progression [5, 6]. Previous studies have shown that lumican is expressed in a variety of malignancies and can promote or inhibit tumor cell proliferation, migration, or invasion, with a positive or negative correlation to tumor progression. In addition, lumican was detected in a variety of cancerous tissues, and it is believed to perform a pro-carcinogenic function in esophageal, colorectal, and lung cancers and a carcinogenic role in breast, pancreatic, and melanoma [7–11]. The regulatory influence of lumican on the proliferation, migration, and invasion of gastric cancer, as well as the molecular processes behind its promoting or inhibiting activities, remain unknown.

Our previous bioinformatics analysis demonstrated that lumican was highly expressed in gastric cancer tissues and significantly enriched for the mitogen-activated protein kinase/extracellular signal regulation (MAPK/ERK) pathway, indicating that lumican may serve as a diagnostic and therapeutic biomarker for gastric cancer via the MAPK/ERK signaling pathway [12]. The MAPK/ERK pathway is extensively involved in tumor signaling and mediates a variety of biological behaviors, including tumor proliferation, invasion, and migration. It functions as a pro-oncogenic signal in tumors as well as a suppressor, with the dominant effect depending on the signal's strength and the environment or tissue in which it is aberrantly activated [13–16]. By downregulating LASP2, for instance, the MAPK/ERK signaling cascade is activated, which enhances the proliferation and migration of breast cancer cells [17]; miR-330-3p promotes cell invasion and metastasis in non-small cell lung cancer [18]. Nevertheless, whether and how lumican affects this pathway in gastric cancer cells remains uncertain.

On the basis of prior research, the purpose of this study was to further analyze the effects of lumican on the proliferation, migration, and invasion of gastric cancer and its molecular pathways, therefore identifying possible novel therapy targets for gastric cancer.

2. Materials And Methods

2.1 Tissues

We collected 22 pairs of GC tissues and adjacent normal tissues from patients at the Affiliated Hospital of Southeast University between May 2015 and May 2020, who were confirmed by pathological testing; their cancerous and paracancerous tissues were surgically removed, and none of the patients had undergone radiotherapy or chemotherapy prior to surgery or had a history of other tumors in combination. All specimens were properly maintained at 80 degrees Celsius until RNA or protein extraction. Prior to inclusion in the trial, informed permission was acquired from each patient. The research design was
approved by the Affiliated Hospital of Southeast University’s Ethics Committee. (approval number: 2014ZDSYLL016.0).

2.2 Cells

Each of the GES-1, HGC-27, MKN-45, AGS, and MGC-803 cell lines has its own STR (short tandem report) report. All cell lines were grown in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco, USA), which includes 10% fetal bovine serum and 1% penicillin and streptomycin. All cells were grown at 37°C with 5% CO₂ [19].

2.3 siRNA and Lentivirus transfection

To knock down lumican, small inhibitory RNAs (siRNAs) targeting the lumican sequence or a negative control were produced by Ruibo (Guangzhou, China) and designated si-lumican and nc-lumican, respectively. A special transfection reagent was used to transiently transfec siRNA into GC cells (Thermofisher, USA). Lentiviral vectors (HBLV-h-LUM-3flag-ZsGreen-PURO) or a negative control were designed for enhancing lumican expression by Hanbio (Shanghai, China) and designated pLV-lumican or pNC-lumican, separately. The multiplicity of infection (MOI) for cell infection was 50. Using quantitative reverse transcription polymerase chain reaction, the infection efficiency was analyzed (qRT-PCR).

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Tissues and cells were extracted using TRizol (GenStar, China) according to the manufacturer's instructions. cDNA concentration was determined using a SYBR Green PCR Kit (GenStar, China). Sangon Biotech (Shanghai, China) developed and manufactured primers containing the following sequences: lumican, 5'- GCTGCCAGAAGACAGTTTGG − 3' (forward) and 5'- GGCACAAGCAACCTATCCAT − 3' (reverse); β-actin, 5'- TCCATCATGAAGTGTGACGT − 3' (forward) and 5'- GAGCAATGATCTTGATCTTCAT − 3' (reverse).

2.5 Western blot (WB)

In RIPA lysis buffer, cells were lysed (RIPA, GenStar, China). The proteins were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Proteins of equal concentrations were separated by 10.5% SDS-PAGE (Bio-Rad, USA) and transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, USA). The membrane was blocked with 5% skim milk powder, the primary antibody was incubated overnight, and the secondary antibody was incubated for 1 hour at room temperature [20]. Chemiluminescent HRP Substrate (Millipore, U.S.) was used to view the blots, and ImageJ was used to analyze them (National Institutes of Health, USA). Western blot antibodies were listed as follows: lumican and β-actin (Abclonal, China), MEK1/2, p-MEK1/2, ERK1/2, and p-ERK1/2 were identified (CST, USA).

2.6 CCK-8 method to detect cell proliferation

CCK-8 technique for measuring cell proliferation GC cells were grown at a density of 5000 cells per unit volume in 96-well plates and treated with 10 L of CCK-8 solution per well after 24, 48, and 72 hours
(Meilun, China). The absorbance of the cells was measured using microplates at 450 nm in accordance with the manufacturer’s instructions (Synergy4; BioTek, USA).

2.7 Transwell assays

In a 24-well plate using Matrigel matrix adhesive, Transwell experiments were conducted (Corning, USA). In 200μl of serum-free media, GC cells were grown in the upper chambers at a density of 8104 cells per unit volume and treated with siRNA or nc-siRNA (RIBO Bio, Guangzhou, China) and pLV-lumican or pNC-lumican (HAN Bio, Shanghai, China). The bottom chambers contained 600μl of a medium containing serum. Following 24 hours of incubation, the cells moving through the well were preserved and stained for 15 minutes with crystal violet (Solarbio, China). The cells were then imaged and counted in three distinct regions.

2.8 Wound-healing assay

The GC cells that had been transfected were put into a 6-well plate and grown until the confluence reached about 100%. The cell surface was then scratched with a pipette tip, and phosphate-buffered saline (PBS) was used to remove cell debris. Images of cells were collected under a microscope at a certain time to analyze the influence of various therapies on the healing of cells (the time for MGC-803 and AGS were both 24 hours, respectively).

2.9 ERK inhibitor research

Ravoxertinib (GDC-0994), a highly selective ERK1/2 inhibitor, was diluted at concentrations of 10, 20, 50, and 100 μm/l for pLV-lumican or pNC-lumican, and the optimum inhibitor concentration was identified 24 hours later using CCK-8. After measuring the OD value at 450 nm with an enzyme marker, cell proliferation was calculated.

2.10 Statistical analysis

For data analysis, Prism 8 software was used. The data were provided as mean standard deviation, and the two samples were independent. To compare data from two groups, the t-test was used. The difference between the means of more than two groups was examined using one-way ANOVA. P values less than 0.05 were considered statistically significant and were shown on graphs.

3. Results

3.1 Lumican was elevated in tissues and cells

Initial tissue investigation revealed an increase in lumican production (Fig. 1a and Table s1, n = 22) Also, the lumican production of five cell lines was evaluated: GES-1, HGC-27, MKN-45, AGS, and MGC-803. In addition, lumican expression was shown to be much greater in HGC-27, MKN-45, AGS, and MGC-803 cells than in GES-1 cells, as evidenced by tissue sample verification data (Fig. 1b and Table s2). Intriguingly, we discovered that HGC-27 was an undifferentiated cell line, MGC-803 and MKN-45 had modest
differentiation, and AGS displayed medium differentiation, as reported at
https://web.expasy.org/cellosaurus (accessed on 15 December 2022) [21]. In addition, AGS was related
with significant lumican expression in MGC-803 cells, and its knockdown was connected with a better
therapeutic benefit; hence, AGS and MGC-803 were chosen for future investigation.

3.2 Knock down and overexpress lumican gastric cancer
cell model construction

Using short interfering RNA (siRNA), two cell lines (MGC-803 and AGS) containing lumican-knockdown
siRNA were generated. Following transfection, nc-lumican was 3.45-fold larger in MGC-803 cells and 1.56-
fold larger in AGS cells compared to si-lumican (Fig. 2a-b, Table s3). To examine the biological function
of lumican in GC cells, lumican was subsequently overexpressed in MGC-803 and AGS cells using a
lumican lentivirus. Following transfection, lumican synthesis was substantially enhanced in MGC-803
and AGS cells, where pLV-lumican levels were 62.38-fold and 18.2-fold greater, respectively, than in pNC-
lumican cells (Fig. 2a-b, Table s3). (P < 0.05)

3.3 The function of lumican in GC cells

CCK-8, Transwell, and wound-healing experiments were conducted using lumican knockdown cell lines
(MGC-803 and AGS). And CCK-8 tests (MGC-803, Fig. 2a and AGS, Fig. 2i), transwell assays (MGC-803,
Fig. 2b-c; AGS, Fig. 2j-k; Table s4), and wound-healing assays (MGC-803, Fig. 2d and AGS, Fig. 2l)
revealed that lumican overexpression boosted in GC cells proliferation, invasion, and migration abilities.
Furthermore, lumican-overexpressing cell lines (MGC-803 and AGS) were effectively established. CCK-8
assays (MGC-803, Fig. 2e and AGS, Fig. 2m), transwell assays (MGC-803, Fig. 2f-g; AGS, Fig. 2n-o; Table
s4), and wound-healing tests (MGC-803, Fig. 2h and AGS, Fig. 2p) demonstrated that lumican
overexpression significantly increased the proliferation, invasion, and migration of GC cells. These data
suggested that lumican significantly promoted the development of cancer. Hence, we hypothesize that
lumican may be involved in the production of GC through unidentified chemical mechanisms.

3.4 The connection between lumican and the MAPK/ERK
signaling pathway

The MAPK/ERK pathway in GC cells may be activated by lumican, as found by our group [22]. As a result,
we used WB to validate in GC cells, as shown knocking down lumican significantly lowered the
expression levels of ERK1/2 and p-ERK1/2 proteins (Fig. 4a-b), while overexpression of lumican had the
opposite effect (Fig. 4b-c). Neither knockdown nor overexpression of lumican significantly affected the
levels of MEK and p-MEK protein expression (Fig. 4a-c). Via the MAPK/ERK pathway proteins ERK1/2 and
p-ERK1/2, but not MEK and p-MEK, lumican has been found to regulate the biological activity of gastric
cancer cells. The optimal concentration (10µm/l) of GDC-0994 dosing was then used to corroborate the
aforementioned results (Fig. 4d-e). After the addition of the ERK inhibitor GDC-0994, the expression of
ERK, p-ERK, and lumican returned to control levels in MGC-803 and AGS cells overexpressing lumican;
MEK and p-MEK expression levels did not change (Fig. 4f-i), demonstrating that lumican regulates ERK1/2 and p-ERK1/2 reciprocally in gastric cancer cells.

4. Discussion

Though due to its metastability and aggressiveness, gastric cancer (GC) has a high mortality rate and is one of the most common malignancies in the world. Early diagnosis and vigorous treatment of GC may significantly improve the survival rate of GC patients. The lumican expression level in GC tissues was higher than that in surrounding tumor tissues, with high lumican expression suggesting a poor prognosis in GC patients and establishing lumican as a biomarker of gastric cancer in a previous bioinformatics study conducted by our lab [22]. Furthermore, the lumican expression level in our clinical data was similar to that in previous bioinformatics studies. XF Wang et al. revealed that cancer-associated fibroblasts (CAFs), which exist in the tumor stroma surrounding tumor cells, are responsible for lumican's contribution to GC development and metastasis [23]. Using novel in vitro functional assays, we discovered that lumican in GC cells controls proliferation, migration, and invasiveness.

In this section, we will concentrate mostly on ERK1/2, the most important participant in the invasion and metastasis of cancer. ERK is associated with hepatocellular carcinoma [24], breast cancer [25], and bladder cancer [26], according to many studies. No investigation has shown the relationship between lumican and the ERK1/2 signaling pathway in GC. Thus, our research provides novel insights into the role of lumican in the genesis and progression of cancer. ERK was also the principal gene involved in proliferation, migration, and invasion [5]. In previous bioinformatics studies done in our lab, the MAPK/ERK signaling pathway was identified as the lumican signaling pathway in GC that is associated with the progression of GC. In addition, these findings revealed that lumican acts through ERK1/2 and p-ERK1/2 in the ERK signaling pathway and that lumican and ERK expression have a reciprocal regulatory role as demonstrated by western blot and revertant experiments, therefore bolstering the therapeutic potential of lumican (Fig. 5). In contrast to other molecules previously regulated by gastric cancer-active genes through the MAPK/ERK signaling pathway [23, 27], this one is novel. This shows that lumican may not need MEK1/2 and p-MEK1/2 activation for ERK1/2 and p-ERK1/2 to exert pro-cancer effects in gastric cancer, the focus of the next research.

Lumican, ERK1/2, and p-ERK1/2 have an essential role in GC proliferation, migration, and invasion metastases, respectively. Lumican increases the proliferation, migration, and invasion of GC cells through increasing ERK expression. Patients with elevated lumican and ERK levels had a poor prognosis. This knowledge may provide a new biomarker and therapeutic target for the diagnosis and treatment of GC.

Declarations

Fundings
This research was funded by a grant from the National Natural Science Foundation of China (No. 81472940).

Declaration of competing interest

The authors state that they have no known conflicting financial interests or personal ties that may be seen as having influenced the work described in this study.

Data availability

All data generated or analyzed during this study are included in this published article.

References


Figure 1

The lumican expression in GC tissues and cells

a. The relative expression of lumican in 22 pairs of gastric cancer tissues (paired sample t-test using the $2^{-\Delta\Delta CT}$ value for each pair of samples); b. qRT-PCR analysis of lumican in five gastric cell lines. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
Figure 2

GC cell models of knockdown and overexpression lumican

a. lumican mRNA levels after lumican was knocked down and overexpressed; b. Western blot findings following lumican knockdown and overexpression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
Figure 3

Lumican stimulated GC cell proliferation, migration, and invasion capacity.

a& e. CCK-8 assay of MGC-803 cells; b& f. Invasion results of the transwell assay of MGC-803 cells; c& g. Migration results of the transwell assay of MGC-803 cells; d& h. Wound-healing assays results of MGC-803 cells; i& m. CCK-8 assay of AGS cells; j&n. Invasion results of the transwell assay of AGS cells; k&o. Migration results of the transwell assay of AGS cells; l&p. Wound-healing assays results of AGS cells; (All samples were imaged at 200* magnification. Scale bar = 100 μm). * P < 0.05, ** P < 0.01, *** P < 0.001
Figure 4

In GC cells, ERK inhibition restored overexpressed lumican, ERK1/2, and p-ERK1/2.

a-c. WB results of lumican, MEK1/2, p-MEK1/2, ERK1/2, and p-ERK1/2 in MGC-803 or AGS cells transfected with mimics-lumican, si-lumican, and their respective control groups; d-e. Screening for appropriate inhibitor-ERK concentrations using the CCK-8 assay; f-i. WB results of lumican, MEK1/2, p-
MEK1/2, ERK1/2, and ERK1/2 in MGC-803 or AGS cells transfected with si-lumican. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Figure 5**

Diagram of lumican and ERK in GC cell formation and metastatic regulation (Created with BioRender.com).
Supplementary Files

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