Original Article
Lipocalin2 promotes proliferation, invasion, migration and glucose metabolism in gastric cancer

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Abstract: Lipocalin2 is differentially expressed and functions in diverse types of cancer. Using bioinformatics software, expression of lipocalin2 was found to be hundreds of times higher in gastric cancer tissues than in normal tissues. Therefore, its function on cell growth, invasion, and migration was assessed by in vitro assays including CCK8, Edu, Transwell, and Boyden assay. Lipocalin2 significantly promoted proliferation, invasion, and migration in gastric cancer cells. Furthermore, cell cycle and EMT showed that lipocalin2 upregulated expression of CCND1, CDK4, CDK6 and N-cadherin, and downregulated E-cadherin, which is consistent with the cell ability assay results. Additionally, glucose and lactate production in gastric cancer cells after lipocalin2 overexpression was measured. Lipocalin2 promoted glucose and lactate concentrations and related protein expression of LDHA and HK2. Taken together, functioning as an oncogene, lipocalin2 promotes progression of gastric cancer including proliferation, invasion, migration, and glucose metabolism, which means lipocalin2 can be a potential therapy target for treatment.

Keywords: Lipocalin2, proliferation, invasion and migration, glucose metabolism, gastric cancer

Introduction

Gastric cancer (GC) is the second leading cause of cancer mortality and the fourth most commonly diagnosed malignant disease with more than 740,000 deaths and 990,000 new definite cases every year [1, 2]. Key proteins trigger progression of gastric cancer, including cell proliferation, invasion and metastasis [3-6]. Many kinds of target-specific policies have been applied in gastric cancer treatment [4, 7], however, fine targeting of drivers of mutations is difficult to achieve with satisfactory outcomes due to tumor heterogeneity of gastric cancer [8]. It is necessary to develop an efficient target therapy for key proteins.

Lipocalin2, also called LCN2, locates on 24p3 and encodes a protein that belongs to the lipocalin family. Because of its special hydrophobic pocket structures, it can transport small hydrophobic molecules such as lipids, steroid hormones and retinoids [9]. The protein encoded by this gene is a neutrophil gelatinase-associated lipocalin and elicits spillover adverse pro-

inflammatory effects in innate immunity by limiting bacterial growth as a result of sequestering iron-containing siderophores [10]. The presence of this protein in blood and urine is an early biomarker of acute kidney injury [11]. This protein is thought to be involved in multiple cellular processes, including playing a protective role in infectious and inflammatory bowel diseases, and both beneficial and detrimental functions have been documented in neurodegenerative diseases, metabolic syndrome, renal disorders and skin disorders [12]. In cancers, lipocalin2 plays dual roles, according to previous studies. In colorectal cancer, lipocalin2 suppresses metastasis and epithelial-mesenchymal transition (EMT) [13]. In cervical cancer, overexpression of lipocalin2 enhances tumor migration and invasion [14]. In ovarian cancer, as a serum biomarker, upregulation of LCN2 is significantly associated with tumor differentiation [15]. In pancreatic ductal adenocarcinoma, lipocalin2 promotes resistance to drug-induced apoptosis, enhance invasion and promote tumor growth [16]. While lipocalin2 is upregulated in gastric carcinoma patients [17],

suggesting its important function in gastric cancer progression.

c-Myc is a proto-oncogene and encodes a nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. In gastric cancer, c-Myc induces G0/G1 cell cycle arrest and promotes migration and invasion [18, 19]. Furthermore, it plays an important role in the regulation of tumor energy metabolism, which can regulate glycolysis to promote the Warburg effect in gastric cancer [20].

In this study, c-Myc was used as a positive control to prove that lipocalin2 promotes proliferation, invasion, and migration in gastric cancer cells by cell assays and Western blot. Specifically, lipocalin2 stimulated glucose and lactate production and increased expression of glucose metabolism proteins LDHA and HK2. Above all, lipocalin2 was defined as an oncogene and a potential therapy target in gastric cancer.

Materials and methods

Cell culture

Gastric cancer cell lines MGC803 and SGC7901 were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). Both cell lines were cultured in RPMI 1640 medium with 10% fetal calf serum (Gibco, Thermo Fisher). Both cell lines were grown in a humidified chamber with 5% CO_2 at 37°C.

Lentivirus infection

Lentivirus carrying lipocalin2 or c-Myc (c-Myc as positive control) sequences was provided by GenePharma Inc, Shangai. MGC803 or SGC7901 cells (1×10^4) were infected with lentivirus or empty controls, respectively. Using Western blot and RT-qPCR technologies, the efficiency of overexpression was assessed.

RNA isolation and RT-qPCR

mRNA of lipocalin2 or c-Myc overexpressed cells and their controls were isolated then reversely transcribed following the instructions of TAKARA, Co., Ltd, Japan. RT-qPCR assay was arranged with direction of SYBR Premix Ex Taq II kit. Primer sequences of Lipocalin2 were 5' CCACCTCAGACCTGATCCCA 3' (Forward Primer) and 5' CCCCTGGAATTGGTTGTCCTG 3' (Reverse Primer). Primer sequences of c-Myc were 5'

GGCTCCTGGCAAAAGGTCA 3' (Forward Primer) and 5' CTGCGTAGTTGTGCTGATGT 3' (Reverse Primer).

Western blot

Western blot technology was used to measure related protein expression. The total proteins were extracted from cells using RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China), loaded and separated on 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Antibody of lipocalin2, c-Myc, CCND1, CDK4, CDK6, GADPH, E-cadherin, N-cadherin, HK2 and LDHA (diluted at 1:1000) were all provided by Abcam, USA.

CCK8 assay

MGC803 and SGC7901 overexpressed cells or their controls were plated into 96-well plates with 2000 cells per well, respectively. After the cells were attached, 20 ul of CCK8 was added (Dojindo, Co., Japan) to each well, with 5 duplications. After incubation for 1 hour, the OD values were detected. The process above was repeated from day 1 to day 7.

Edu assay

MGC803 and SGC7901 overexpressed cells and their controls were plated into 96-well plates and fixed by methanol for one hour. Following the instructions of Edu kit of Ribo Bio Inc, China, cells in S phase were dyed red. Cell numbers and the percentage in S phase were counted and analyzed.

Transwell and Boyden assay

A total of 10^5 cells were plated into Transwell or Boyden chambers, then fixed by methanol and stained with Giemsa (AppliChem, Germany) after 16 hours. Cell numbers were used to assess invasion and migration abilities of the cells.

Wound healing assay

Cells were plated into a 6-well plate at 80% density in RPMI 1640 medium with 1% FBS; a wound was swept in the middle of the plate by a tip. The width of the wound after 72 hours was observed and compared with controls.

Glucose metabolism assay

Glucose and lactate assay kit were purchased from Biovision, USA. According to the instructions, glucose or lactate reacts specifically with
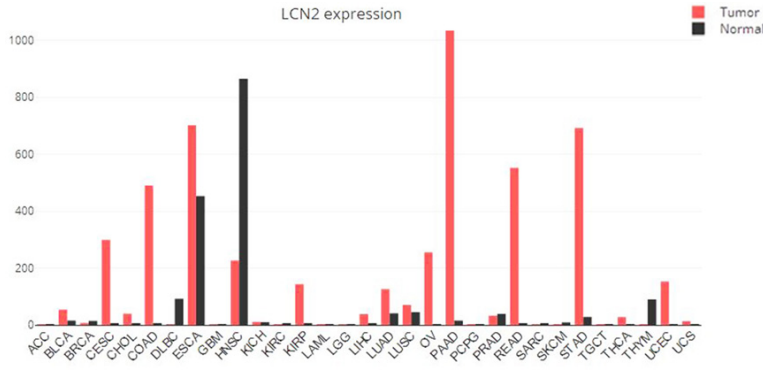
Figure 1. Expression of lipocalin2 in tumors and normal tissues. STAD: Stomach adenocarcinoma.
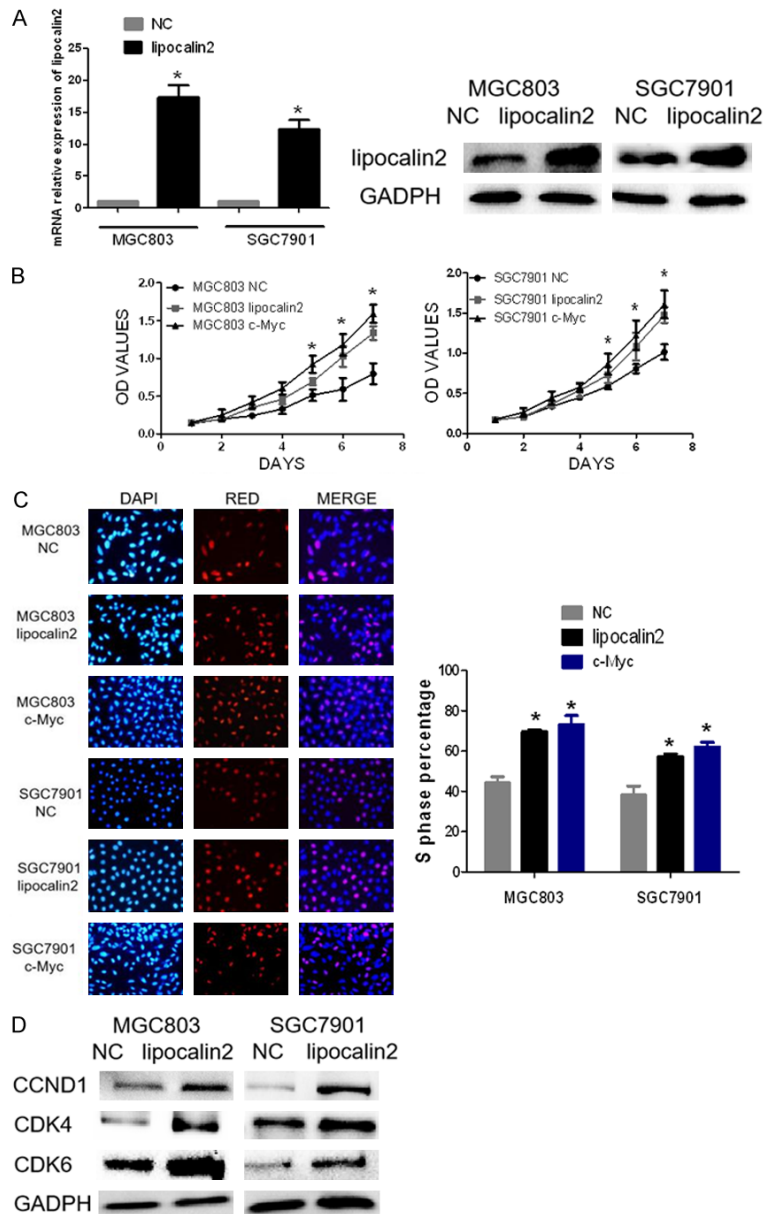

Figure 2. Lipocalin2 promotes proliferation in gastric cancer. A. Expression of lipocalin2 overexpressed measured by qRT-PCR and Western blot. B. Cell growth measured by CCK8 after lipocalin2 overexpressed. C. S phase percentage measured by Edu after lipocalin2 or c-Myc overexpressed. D. Levels of CCND1, CDK4, CDK6 after lipocalin2 overexpressed detected by western blot, GADPH as internal reference. P<0.05. Experiments were repeated three times.

the enzyme mixture and forms a product that interacts with glucose or lactate probe. OD value was used to estimate glucose or lactate concentration.

Statistical analysis

All quantified data represented an average of at least triplicate samples. GraphPad Prism v6.0 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for statistical analysis. Data are presented as mean ± SEM. One-way analysis of variance or two-tailed Student's t-test was used for comparisons between groups. Fischer's or χ^2-test was used to identify differences between categorical variables. Partial correlations were applied in multivariate correlations analyses. Differences were considered statistically significant when $P<0.05$.

Results

Lipocalin2 is highly expressed in gastric cancer

To discern lipocalin2 expression in gastric cancer, bioinformatics online software GEPIA (Gene Expression Profiling Interactive Analysis) was used to analyze expression of lipocalin2 in different kinds of tumors. As shown in **Figure 1**,
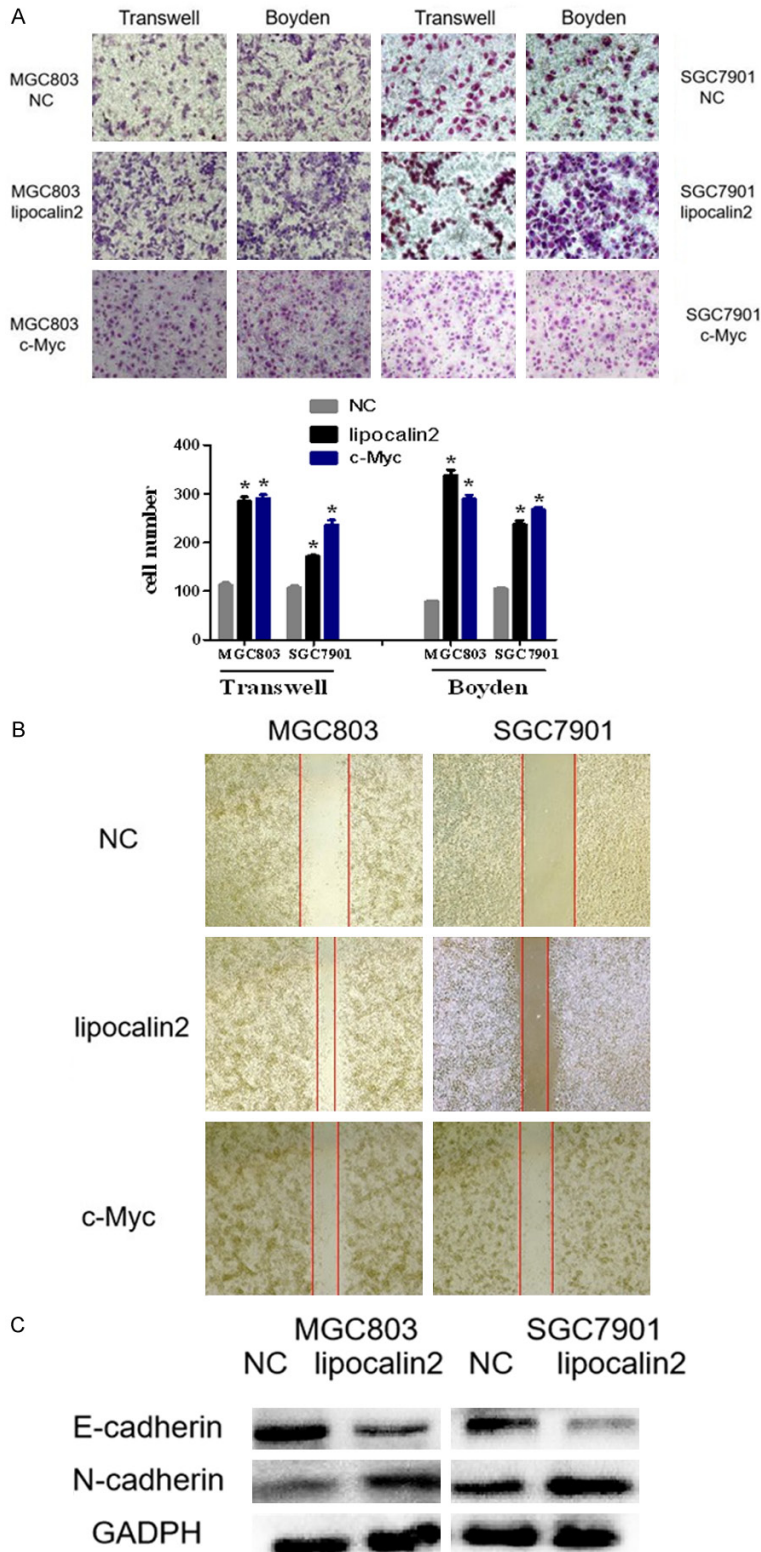
Figure 3. Lipocalin2 promotes invasion and migration in gastric cancer. A-B. Change of cell invasion and migration assessed by Transwell and Boyden Chamber (A) and Would Healing assays (B) after lipocalin2 or c-Myc overexpressed. (C) Expression of E-cadherin and N-cadherin after lipocalin2 overexpressed, GADPH as internal reference. P<0.05. Experiments were repeated three times.

expression of lipcalin2 exhibited different levels in different kinds of cancers, suggesting its distinct functions in different cancers. In gastric cancer (represented as STAD), the level of lipocalin2 was hundreds of fold higher than in normal tissues, suggesting lipocalin2 might play an oncogene role in gastric cancer.

Lipocalin2 promotes proliferation in gastric cancer

To study the biological function of lipocalin2 in gastric cancer, two gastric cancer cell lines, MGC803 and SGC7901, were generated to stably overexpress lipocalin2 or c-Myc with lentivirus, and c-Myc was used as a positive control. Western blot and RT-qPCR technologies were used to assess the efficiency of overexpression (**Figure 2A**). Then CCK8 and Edu assays were used to evaluate the effect of lipocalin2 on cell proliferation. The CCK8 results showed (**Figure 2B**) cells transfected with lipocalin2 lentivirus grew faster than the negative control, and there was no significant difference between the lipocalin2 group and the c-Myc group. Furthermore, Edu assays results showed that S phase percentage in lipocalin2 overexpressed cells were higher than in negative controls (**Figure 2C**). Using Western blot, cell cycle factors including CCND1, CDK4 and CDK6 were examined, finding that lipcalin2 markedly upregulated protein level of CCND1, CDK4 and CDK6 in both cell groups (**Figure 2D**).

Lipocalin2 promotes invasion and migration in gastric cancer

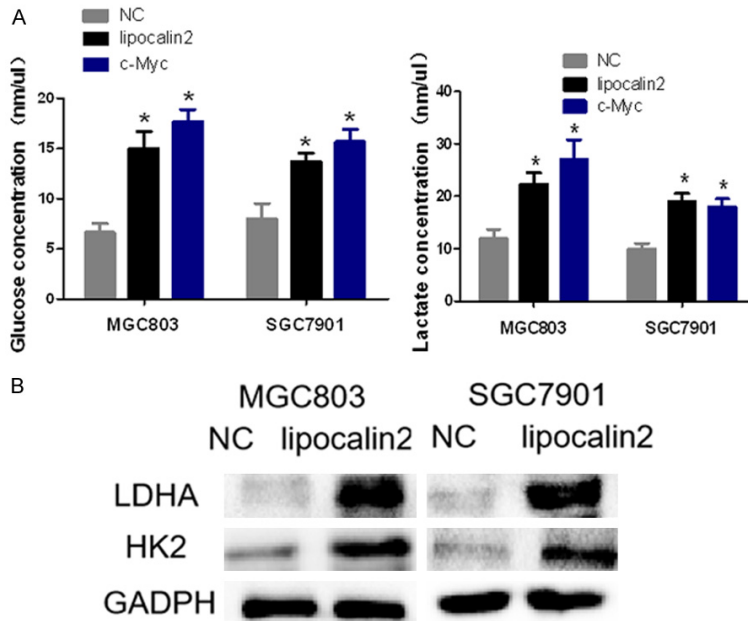Next, Transwell and Boyden and Wound Healing assays

Figure 4. Lipocalin2 promotes glucose metabolism in gastric cancer. A. Change of glucose and lactate concentrations after lipocalin2 or c-Myc overexpressed. B. Expression of LDHA and HK2 detected by Western blot after lipocalin2 overexpressed, GADPH as internal reference. P<0.05. Experiments were repeated three times.

were performed to assess the influence of lipocalin2 on cell invasion and migration. As described in **Figure 3A**, cell numbers in lipocalin2 overexpressed cells were significantly higher than in controls, meaning lipcalin2 promotes invasion and migration in gastric cancers. Additionally, wound healing assays showed cells with lipocalin2 overexpression had a stronger ability to heal the wound than controls after 72 hours (**Figure 3B**). EMT factors including E-cadherin and N-cadherin were detected after lipcalin2 overexpression, finding lipcalin2 increased expression of N-cadherin and decreased levels of E-cadherin (**Figure 3C**), also meaning lipcalin2 promoted invasion and migration in gastric cancer.

Lipocalin2 promotes glucose metabolism in gastric cancer

There is a study that demonstrates that mice lacking lipocalin2 have compromised glucose metabolism with decreased glucose tolerance and insulin sensitivity [18], suggesting that lipocalin2 might affect glucose metabolism. To discover whether lipocalin2 functions on glycolysis in gastric cancer, assays were arranged to detect levels of glucose and lactate production in gastric cancer cells with lipocalin2 overex-

pression. The results are displayed in **Figure 4A** where both MGC803 and SGC7901 with overexpressed lipocalin2 had higher glucose and lactate concentrations than their controls. To further confirm this result, glycolysis related enzymes, LDHA and HK2, were examined by Western blot. Lipocalin2 upregulated expression of LDHA and HK2, which was consistent with the glucose metabolism production assays (**Figure 4B**).

Discussion

Lipocalin2 is a controversial protein for its dual roles in cancers, such as facilitating tumor growth in breast cancer [13, 19, 20], while suppressing metastasis in colorectal cancer. In gastric cancer, with c-myc as a positive control, lipocalin2 was found to have enhanced cell proliferation by upregulating key cell cycle proteins, CCND1, CDK4, and CDK6, which are essential for G1/S phase transition. Edu assay also identified that cells with lipocalin2 overexpression had higher percentage of S phase compared to controls. Therefore, lipocalin2 promoted cell division from G1 phase to S phase, improving cancer cell survival.

EMT is a crucial process in tumor progression that causes epithelial cells to acquire fibroblast-like properties and have reduced intercellular adhesion and increased motility, which is recognized as a critical event during carcinoma migration and invasion [21, 22]. Similar to findings in cervical cancer and pancreatic ductal adenocarcinoma, lipocalin2 significantly enhanced cell invasion and migration abilities. Also, overexpression of lipocalin2 upregulated levels of N-cadherin and downregulated E-cadherin.

Malignant tumors usually consume glucose for their high metabolism demands [23]. Suppression of glucose consumption can effectively depress cancer cell progression [24]. In this study, lipocalin2 increased glucose uptake, lactate production and the expression of LDHA

and HK2 involved in glucose metabolism, further confirming the oncogene role of lipocalin2 in gastric cancer. Inhibition of lipocalin2 could suppress glucose metabolism to disturb the malignant process, which needs verification.

Lipocalin2 is an oncogene that notably accelerates the tumor development process, suggesting it could be a potential biomarker and therapeutic target for gastric cancer.

Disclosure of conflict of interest

None.

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