SHORT REPORT

**LncRNA FGD5-AS1 drives the malignant development of gastric cancer by negatively interacting with FZD3**

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We aimed to detect the expression pattern of long non-coding RNA (lncRNA) FGD5-AS1 in gastric cancer (GC) samples and its impact on driving the development of GC. FGD5-AS1 levels in 66 cases of GC tissues and paracancerous ones were detected. Its influences on clinical features and prognosis in GC patients were analyzed. In AGS and SGC-7901 cells with FGD5-AS1 knockdown, phenotype changes were assessed through cell counting kit-8 (CCK-8), Transwell and wound healing assay. The downstream target of FGD5-AS1 was searched by a bioinformatics tool and confirmed by dual-luciferase reporter assay. Their interaction in regulating the malignant development of GC was finally explored. FGD5-AS1 was upregulated in GC tissues compared to paracancerous ones. GC patients expressing a high level of FGD5-AS1 had higher risk of lymphatic metastasis or distant metastasis and worse prognosis than those with a low level. Knockdown of FGD5-AS1 weakened proliferative and metastatic abilities in AGS and SGC-7901 cells. FZD3 was the downstream target of FGD5-AS1. Protein levels of FZD3 and FZD5 were upregulated, while β-catenin, TGF-β and MMP9 were downregulated in GC cells with FGD5-AS1 knockdown. Knockdown of FZD3 abolished the regulatory effects of FGD5-AS1 on malignant phenotypes of GC cells. FGD5-AS1 is upregulated in GC samples, which is linked to metastasis and prognosis in GC. It drives proliferative and metastatic abilities in GC cells via negatively interacting with FZD3.

**Key words:** FGD5-AS1, FZD3, gastric cancer (GC), malignant development.

Introduction

Gastric cancer (GC) is the third most fatal malignant tumor in the world. Its incidence is high in Asia [1, 2]. In recent years, although the incidence of GC has gradually decreased owing to advanced research and lifestyle improvement, as many as 723,000 people die of GC [1, 3, 4]. The prognosis in advanced GC patients or those with metastases is very poor [4, 5]. Currently, radical resection is the major therapeutic strategy for GC. In the meantime, targeted treatment is preferred as an adjuvant way for those with a poor response to surgery, chemotherapy and/or radiotherapy [2, 5]. Its therapeutic efficacy displays a promising outcome in clinical treatment of many types of malignancies [6, 7]. However, GC-specific molecules are largely unknown [7, 8]. Non-coding RNAs, which have been identified in recent years, are considered to be promising tumor targets [9, 10].

As genome studies revealed, the number of protein-encoding genes accounts for less than 3% of total genes, and over 80% are transcribed into non-coding RNAs [10, 11]. They are classified according to the length [12, 13]. Those over 200 nu-
cleotides long are known as long non-coding RNAs (lncRNAs) [14, 15]. Functionally, lncRNAs are able to regulate gene expression at epigenetic, transcriptional and post-transcriptional levels [15, 16]. The latest evidence has demonstrated that abundantly expressed lncRNAs in the human genome may serve as oncogenes or tumor suppressors influencing tumor development [16, 17]. They act as baits, signaling molecules or scaffolds in the regulation of cell behaviors, thus exerting diagnostic and therapeutic potential in tumors [17].

Great progress has been made in screening and identifying lncRNAs [12, 13]. Microarray analyses and high-throughput sequencing have motivated the findings of tumor-associated lncRNAs [15, 16, 17]. Here, we searched differentially expressed lncRNAs in diffuse-type GC profiling and analyzed them, and finally, lncRNA FGD5-AS1 was selected to be mainly explored. Previous studies have shown the cancer-promoting effects of FGD5-AS1 on lung cancer and oral cancer [18, 19]. In this paper, we first detected the expression pattern of FGD5-AS1 in GC tissues collected in our center. Its influences on clinical features of GC patients and malignant phenotypes of GC cells were subsequently illustrated.

Material and methods

GC patients

We collected 66 pairs of diffuse-type GC (poorly differentiated adenocarcinoma) and paracancerous tissues during surgery. Patients with two or more different types of tumors were excluded. None of them had pre-operative anti-cancer treatment. All collected tissue samples were pathologically confirmed and stored at −80°C. This study was in line with the Declaration of Helsinki clinical practice guidelines. This study obtained approval from the Ethics Committee of Yantai Yuhuangding Affiliated Hospital of Qingdao University and it was conducted after receiving informed consent from each subject.

Cell lines and reagents

GC cell lines (AGS, BGC-823, SGC-7901, MGC-803, HGC-27 and MKN45) and epithelial cells of gastric mucosa (GES-1) purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) in a 5% CO2 incubator at 37°C. 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin were applied in culture medium.

Transfection

Cells were inoculated in 6-well plates and cultured to 30-40% confluence. They were transfected with plasmids constructed by GenePharma (Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was tested by quantitative real-time polymerase chain reaction (qRT-PCR) at 48 hours.

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in a 96-well plate with 2 × 10³ cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

Transwell assay

200 μl of suspension (5.0 × 10⁵/ml) was applied in the upper side of the Transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 460 μl of medium containing 10% FBS in the bottom. After 48 hours of incubation, cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 20 min and counted using a microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 40×). Invasion assay was similarly conducted where the chambers were pre-coated with diluted Matrigel.

Wound healing assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. 24 hours later, wound closure was captured for calculating the percentage of wound healing (magnification 40×).

QRT-PCR

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR Premix Ex Taq (TaKaRa, Otsu, Japan). Each sample was performed in triplicate, and the relative level was calculated by 2⁻ ΔΔ Ct and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). FGD5-AS1: Forward: 5’-GAAGGGCCGCAAGAGCTCAAT-3’, Reverse: 5’-GGCTCGCAAAGTGTCTGTTG-3’; FZD3: Forward: 5’-GCAGAGAATATCACATTCCATCT-3’, Reverse: 5’-CGCTCCTATTTGTATGGAATACT-3’; GAPDH: Forward: 5’-TATGATGATATCAAGAGGTTAGT-3’, Reverse: 5’-TGTATCCAAACTCTTGTGCTCATAC-3’.
Western blot

Cells were lysed on ice for isolating proteins. After detection of protein concentration by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China), protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). They were subsequently loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for the indicated time. Band exposure and analyses were finally conducted.

Dual-luciferase reporter assay

Cells were inoculated in 24-well plates. On the next day, they were co-transfected with FGD5-AS1-WT/FGD5-AS1-MUT and NC/pcDNA-FZD3. After 48 hours cell culture, cells were lysed for measuring luciferase activity (Promega, Madison, WI, USA).

Statistical analyses

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the t-test. The influences of FGD5-AS1 on clinical data of GC patients were analyzed by χ² analysis. The Pearson correlation test was conducted for analyzing the relationship between relative expressions of FGD5-AS1 and FZD3 in GC tissues. Kaplan-Meier curves were depicted for survival analysis. P < 0.05 was considered as statistically significant.

Results

FGD5-AS1 was highly expressed in GC

We collected 66 pairs of GC and paracancerous tissues in our center. QRT-PCR data showed higher abundance of FGD5-AS1 in GC tissues than controls (Fig. 1A, 1B). Meanwhile, it was upregulated in GC cell lines as well (Fig. 1C).

Fig. 1. FGD5-AS1 was highly expressed in GC. (A) FGD5-AS1 level in GC and paracancerous tissues. (B) FGD5-AS1 levels in 12 pairs of GC and normal tissues. (C) FGD5-AS1 level in GC cell lines. (D) Overall survival in GC patients based on their levels of FGD5-AS1. Data are expressed as mean±SD; *p < 0.05, **p < 0.01, ***p < 0.001
Fig. 2. Knockdown of FGD5-AS1 inhibited proliferative and metastatic abilities in GC. A) Transfection efficacy of sh-FGD5-AS1 in AGS and SGC-7901 cells. B) Viability at day 1 to day 4 in AGS and SGC-7901 cells transfected with sh-NC or sh-FGD5-AS1. C) Migration and invasion in AGS and SGC-7901 cells transfected with sh-NC or sh-FGD5-AS1 (magnification: 40×). D) Wound closure percentage in AGS and SGC-7901 cells transfected with sh-NC or sh-FGD5-AS1 (magnification: 40×). Data are expressed as mean±SD.; *p < 0.05, **p < 0.01
Fig. 3. FZD3 was the direct target of FGD5-AS1. A) Luciferase activity in AGS and SGC-7901 cells co-transfected with FGD5-AS1-WT/FGD5-AS1-MUT and NC/pcDNA-FZD3. B) Protein levels of FZD3, FZD5, β-catenin, TGF-β and MMP9 in AGS and SGC-7901 cells transfected with sh-NC or sh-FGD5-AS1. C) FZD3 level in GC and paracancerous tissues. (D) A negative correlation between relative expression of FGD5-AS1 and FZD3. E) Overall survival in GC patients based on their levels of FZD3. F) FZD3 level in GC cell lines. Data are expressed as mean±SD; *p < 0.05, **p < 0.01, ***p < 0.001
FGD5-AS1 expression was correlated with metastasis and overall survival in gastric cancer patients

Based on the mRNA levels of FGD5-AS1 in 66 collected GC tissues, recruited GC patients were assigned to two groups based on the median level of FGD5-AS1. By analyzing clinical data of them, it was found that FGD5-AS1 level was positively correlated to risks of lymphatic metastasis and distant metastasis in GC patients. Its level, however, was unrelated to age, sex and tumor staging in GC (Table I). Their follow-up data were recorded for depicting Kaplan-Meier curves. As shown in Fig. 1D, a high level of FGD5-AS1 was unfavorable to the survival in GC.

Knockdown of FGD5-AS1 inhibited proliferative and metastatic abilities in GC

To explore the biological functions of FGD5-AS1 in GC cells, the FGD5-AS1 knockdown model was established in AGS and SGC-7901 cells by transfection of sh-FGD5-AS1 (Fig. 2A). Compared with those transfected with sh-NC, viability was markedly lower in GC cells with FGD5-AS1 knockdown (Fig. 2B). Migratory and invasive cell numbers decreased in GC cells transfected with sh-FGD5-AS1 (Fig. 2C). In addition, a lower percentage of wound closure was found in GC cells transfected with sh-FGD5-AS1 than in controls (Fig. 2D). It was concluded that FGD5-AS1 stimulated proliferative and metastatic abilities in GC.

FZD3 was the direct target of FGD5-AS1

To verify the binding effect of FZD3 on FGD5-AS1, dual-luciferase reporter assay was conducted. It showed that FGD5-AS1 could be targeted by FZD3 (Fig. 3A). Protein levels of FZD3 and FZD5 were upregulated in GC tissues compared to paracancerous ones. Meanwhile, a high level of FGD5-AS1 was upregulated in GC tissues (Fig. 3C). In GC tissues, FGD5-AS1 level was negatively correlated with that of FZD5 (Fig. 3D). Kaplan-Meier curves demonstrated that lowly expressed FZD5 predicted a poor prognosis in GC patients (Fig. 3E). Conversely to the expression pattern of FGD5-AS1 in GC, FZD3 was downregulated in GC cells (Fig. 3B). Protein level of FZD3 was effectively downregulated after transfection of si-FZD3 in AGS and SGC-7901 cells with FGD5-AS1 knockdown (Fig. 4A). As the CCK-8 curves showed, viability was higher in GC cells with co-silence of FGD5-AS1 and FZD3 than those with FGD5-AS1 knockdown (Fig. 4B). As expected, FZD3 knockdown reversed the inhibitory effects of silenced FGD5-AS1 on migratory and invasive potentials in GC (Fig. 4C).

Discussion

GC is a highly malignant disease ranking as the third cause of death from cancer in the world [1, 2, 3]. Although improved therapeutic strategies contribute to enhancing the control rate of local foci and decrease the distant metastasis rate, the prognosis in progressive GC is far from satisfactory [4, 5, 6]. The lack of early screening methods and effective treatment for advanced GC explains the high mortality of GC [5, 6]. Therefore, it is urgent to develop specific hallmarks, hierarchical screening mechanisms and individualized treatment for GC [7, 8]. Modern biomedical research has explored many potential GC biomarkers by examining serum protein antigens, exploring biological functions of tumor-associated genes and sequencing them [8]. However, there is still room for improving the specificity, and processability of the molecular diagnosis [6, 7, 8]. Recent studies have highlighted the diagnostic potential of lncRNAs in human tumors [9, 10].

LncRNAs are non-coding RNAs containing over 200 nucleotides [11, 12]. They are extensively involved in biological processes. A previous study demonstrated that the oncogene FGD5-AS1 drives NSCLC proliferation through the miR-107/FGFRL1 axis [18]. In addition, it is able to stimulate the proliferative and metastatic potential in colorectal cancer via competitively binding miR-302e to upregulate CDC7 [20]. Our findings revealed that FGD5-AS1 was upregulated in GC tissues compared to paracancerous ones. Meanwhile, a high level of FGD5-AS1 predicted high metastasis risk and poor prognosis in GC patients. We therefore believe that FGD5-AS1 is an oncogene involved in the malignant development of GC. Subsequently, an FGD5-AS1 knockdown model was established in GC cells by transfection of sh-FGD5-AS1. Experimental evidence has shown that knockdown of FGD5-AS1 markedly weakened viability, metastatic potential and wound healing ability in AGS and SGC-7901 cells. It is suggested that FGD5-AS1 was responsible for driving proliferative and metastatic abilities in GC.

Generally speaking, lncRNAs exert their biological functions through acting as miRNA sponges (the ceRNA hypothesis) or binding proteins. LncRNA-mRNA interaction is rarely reported [21, 22]. Bioinformatics analysis showed that FGD5-AS1 could bind FZD3, which was confirmed by the dual-
Fig. 4. FGD5-AS1/FZD3 axis was responsible for the malignant phenotypes of GC. A) Protein level of FZD3 in AGS and SGC-7901 cells co-transfected with sh-NC+si-NC, sh-FGD5-AS1+si-NC or sh-FGD5-AS1+si-FZD3. B) Viability at day 1 to day 4 in AGS and SGC-7901 cells co-transfected with sh-NC+si-NC, sh-FGD5-AS1+si-NC or sh-FGD5-AS1+si-FZD3. C) Migration and invasion in AGS and SGC-7901 cells co-transfected with sh-NC+si-NC, sh-FGD5-AS1+si-NC or sh-FGD5-AS1+si-FZD3 (magnification: 40×). Data are expressed as mean±SD. *P < 0.05.
luciferase reporter assay we conducted. Conversely to the expression pattern of FGD5-AS1 in GC, FZD3 was downregulated in GC samples and negatively regulated by FGD5-AS1. Importantly, knockdown of FZD3 reversed the inhibitory effects of silenced FGD5-AS1 on the malignant phenotypes of GC cells. The above evidence indicated that the transcriptional activity of the gene locus where FGD5-AS1 was located may be regulated by FZD3, thereby driving proliferative and metastatic abilities in GC.

FGD5-AS1 is upregulated in GC samples, which is linked to metastasis and prognosis in GC. It drives proliferative and metastatic abilities in GC cells via negatively interacting with FZD3.

The authors declare no conflict of interests.

References