LncRNA FGD5-AS1 functions as an oncogene to upregulate GTPBP4 expression by sponging miR-873-5p in hepatocellular carcinoma

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The long non-coding FGD5-AS1 (LncFGD5-AS1) has been reported to be a novel carcinogenic gene and participant in regulating tumor progression by sponging microRNAs (miRNAs). However, the pattern of expression and the biological role of FGD5-AS1 in hepatocellular carcinoma (HCC) remains largely unknown. The expression level of FGD5-AS1 in tumor tissues and cell lines was measured by RT-qPCR. CCK-8, EdU, flow cytometry, wound healing and transwell chamber assays were performed to investigate the role of FGD5-AS1 in cell proliferation, apoptosis, migration, and invasion in HCC. Dual luciferase reporter, and RNA pull-down assays were performed to identify the regulatory interactions among FGD5-AS1, miR-873-5p and GTP-binding protein 4 (GTPBP4). We found that the expression of FGD5-AS1 was upregulated in HCC tissues and cell lines. Moreover, the knockdown of FGD5-AS1 suppressed cell proliferation, migration and invasion, and induced apoptosis in HCC cells. Further studies demonstrated that FGD5-AS1 could function as a competitive RNA by sponging miR-873-5p in HCC cells. Moreover, GTPBP4 was identified as direct downstream target of miR-873-5p in HCC cells and FGD5-AS1 mediated the effects of GTPBP4 by competitively binding with miR-873-5p. Taken together, this study demonstrated the regulatory role of FGD5-AS1 in the progression of HCC and identified the miR-873-5p/GTPBP4 axis as the direct downstream pathway. It represents a promising novel therapeutic strategy for HCC patients.

Key words: Hepatocellular carcinoma; FGD5-AS1; miR-873-5p; GTPBP4.

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. According to the 2020 Global Cancer Statistics, about 906,000 new cases of liver cancer were diagnosed and 830,000 people died of liver cancer worldwide that year. The efficacy of radiotherapy and chemotherapy is limited and the current surgical treatment for HCC is effective only at the initial stage, but most patients grow into advanced stages after diagnosis. Therefore, it is necessary to further understand the molecular mechanism of liver cancer progression and develop more effective therapies for liver cancer.

In recent years, growing evidence has shown that lncRNAs act as biomarkers for the diagnosis and prognosis of cancer patients, providing new therapeutic targets for cancer treatment. An increasing number of reports have found that aberrant lncRNA expression plays a critical role in hepatocarcinogenesis and metastasis. For instance, MALAT1 is associated with tumor metastasis and predicts recurrence after liver transplantation. Furthermore, lncRNA-LET inhibits hepatic invasion and abdominal metastasis. FGD5 antisense RNA 1 (FGD5-AS1) is a novel identified lncRNA located at 3p25.1. Recently, FGD5-AS1 was newly identified as a potential therapeutic target for HCC. However, the role of FGD5-AS1 in the regulation of HCC remains unknown.

Materials and Methods

Human samples

Tumor tissues and paired adjacent normal tissues were obtained from 60 HCC patients who underwent surgery at the Second Affiliated Hospital of Nanchang University. All the tissue samples were collected and frozen in the liquid nitrogen, then stored at -80°C to further use.

Cell culture

Four human HCC cell lines, including Hep G2, HuH-7, Li-7 and SNU-387, and the normal human liver cell line L02 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C with 5% CO2.

Cell transfection

Hep G2 and HuH-7 cells were cultured in 6-well plates at 40% confluence and transfected with FGD5-AS1 over-expressed vector, FGD5-AS1 shRNA (sh-FGD5-AS1), NC shRNA (sh-NC) and GTPBP4 shRNA (sh-GTPBP4). NC mimic or miR-873-5p mimic, synthesized by GenePharma Co, Ltd. (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the manufacturer’s protocols. RT-qPCR and green fluorescence microscopy were performed to assess the transfection efficiency.

CCK-8 and EdU assays

Cell proliferation was detected by CCK-8 and EdU assays. The CCK-8 assay was performed according to the manufacturer’s instructions (Beyotime, Shanghai, China). The optical density (OD) values were then measured at 450 nm. Cell proliferation was also determined by EdU assay using an EdU Apollo DNA in vitro kit (RIBOBIO, Guangzhou, China) following the manufacturer’s instructions. The cells were visualized under a fluorescence microscope.

Wound healing assay

The scratch wounds were created in Hep G2 and HuH-7 cells using pipette tips. Cells were washed with medium and allowed to migrate for 24 h in culture settings. After migration, representative images were photographed using a microscope (200× magnification; Leica, Wetzlar, Germany).

Flow cytometry

To measure cell apoptosis, Annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA, USA) was applied. Cells were seeded into 24-well plates with medium and cultured for 24 h. After then, cells were digested, centrifuged in 300 g at 4°C for 5 min and transferred to a new centrifuge tube. Subsequently, cells were resuspended in ice cold PBS and centrifuged in 300 g at 4°C for 5 min. After removal of PBS, a total of 100 µl 1x binding buffer was added to each tube and thoroughly mixed. Following incubation with Annexin V-FITC and PI for 15 min in the dark according to the manufacturer’s instruction. Cells were added with 400 µl of binding buffer and incubated on ice to be detected within 1 h using a flow cytometer (BD Biosciences).

Transwell chamber assay

Cells in serum-free medium were seeded in the upper chamber (Cell Biolabs, Inc., San Diego, CA, USA) with Matrigel-coated membrane for invasion assays or in chambers not coated with Matrigel for migration assays, while the lower chamber was filled with complete medium as a chemoattractant. After 24 h, these cells on the upper part of the filters were scrubbed with a cotton swab. After staining with 0.5% crystal violet, the migrated and invaded cells were counted in 5 different fields (200× magnification).
Dual luciferase reporter assay

Both Hep G2 and HuH-7 cells were transfected with either GTPBP4 wild type (WT) or mutated-type (Mut) promoter reporters in combination with FGD5-AS1 or miR-873-5p mimic. 48 h after transfection, the luciferase activities were detected by using the Dual Luciferase Reporter Assay System (Promega, WI, USA).

RNA pull-down assay

Pierce™ Magnetic RNA-Protein Pull-Down Kit (#20164, ThermoFisher Scientific, Waltham, MA, USA) was used for the RNA pull-down assay. RNA was first bound to the beads to receive the RNA for protein binding. Next, the RNA-bound beads were balanced in Protein-RNA Binding Buffer before the addition of the protein lysate. The beads were then washed by adding the appropriate buffer, separating after vortexing on a magnetic stand. Finally, RT-qPCR assay was used to assess the specificity of RNAs in precipitates.

RT-qPCR assay

For the qPCR assay, total RNA was extracted from tissue samples and cells using TRIzol reagent (Invitrogen). RNA was reverse-transcribed into first-strand cDNA using PrimeScript™ RT reagent Kit (Takara, Osaka, Japan) according to the manufacturer’s protocols. The PCR amplification products were quantified by the FastStart Universal SYBR Green Master (Roche) and normalized to GAPDH and U6. PCR cycling condition was according to the previous description. The RNA expression was assessed using 2-ΔΔCt as previously described.

Western blot assay

Total protein was extracted using RIPA buffer (Beyotime, Shanghai, China) which contained protease and phosphotase inhibitors cocktails. BCA protein assay kit (Beyotime) was used to determine protein concentrations according to the manufacturer’s instructions. Proteins were separated by SDS-PAGE, transferred to PVDF (Millipore, Burlington, MA, USA) membranes, incubated with primary antibodies overnight at 4°C and then incubated with HRP-conjugated secondary antibody (1:5000 dilution) for 1 h in room temperature. Proteins were visualized by using ECL western blotting detection reagents (Millipore). Immunoreactive bands were quantified using ImageJ (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism and all data were presented as mean ± standard deviation (SD) from at least three independent experiments. Differences between groups were analyzed by unpaired t-test, one-way ANOVA and two-way ANOVA. To classify and indicate significant values, the following p-values were used: *p<0.05, **p<0.01, ***p<0.001; n.s. indicates not significant.

Results

FGD5-AS1 is highly expressed in HCC tissues and cells

To investigate the role of FGD5-AS1 in HCC, we first examined the expression of FGD5-AS1 by RT-qPCR in tumor tissues and corresponding adjacent non-cancer tissues. As shown in Figure 1A, the RNA expression level of FGD5-AS1 was significantly increased in the HCC tissues when compared with para-carcinoma liver tissues. Consistent with the clinical results, the RNA expression level of FGD5-AS1 was elevated in four HCC cell lines (Hep G2, HuH-7, Li-7 and SNU-387) when compared with normal liver cell line L02. All of this data indicates a positive relationship between FGD5-AS1 and HCC progression.

Knockdown of FGD5-AS1 represses HCC cell proliferation and induces apoptosis

To determine whether FGD5-AS1 affected tumor progression, we performed loss-of-function assays and the specific shRNA targeting FGD5-AS1 was used to stably knockdown FGD5-AS1 expression both in Hep G2 and HuH-7 HCC cells. The knockdown efficiency was presented in Figure 2A and 2B, the expres-
Expression level of FGD5-AS1 was reduced by 70% in HepG2 cell line and by 65% in HuH-7 cell line after transfection with sh-FGD5-AS1. CCK-8 and EdU assays revealed that knockdown of FGD5-AS1 inhibited the proliferation of Hep G2 and HuH-7 HCC cell lines (Figure 2C-D). Likewise, Western blot analysis illustrated that knockdown of FGD5-AS1 resulted in decreased levels of proliferation-associated markers PCNA and CyclinD1 (Figure 2E). The effects of FGD5-AS1 on cell apoptosis were examined by flow cytometry and the results revealed that depletion of FGD5-AS1 increased the percentage of Annexin V-positive cells from 8 to 24% in HepG2 cells and from 7 to 20% in HuH-7 cells (Figure 2F). To further confirm the effects of FGD5-AS1 on the apoptosis in HCC cells, the protein levels of four apoptotic markers (Bax, Bcl-2, caspase-3 and caspase-9) were detected by Western blot assay. As shown in Figure 2G, silencing FGD5-AS1 lessened Bcl2 expression level while increasing the levels of Bax, caspase-3 and caspase-9 both in Hep G2 and HuH-7 cell lines. Based on the above findings, we concluded that FGD5-AS1 knockdown suppressed proliferation and induced apoptosis of HCC cells.
Knockdown of FGD5-AS1 represses migration and invasion of HCC cells

As cell migration and invasion are two critical steps for the cancer metastasis, we also assessed the effects of FGD5-AS1 on migration and invasion of HCC cells. We found that reduction of FGD5-AS1 significantly inhibited HCC cell migration and invasion as evidenced by the wound healing and transwell chamber assays (Figure 3 A,B). At the molecular level, we measured the expression levels of migration/invasion-associated proteins, Cox-2, MMP2 and MMP9. It was found that the protein expression levels of Cox-2, MMP2 and MMP9 in sh-FGD5-AS1 group were lower than those in sh-NC group (Figure 3C). In short, inhibition of FGD5-AS1 restrained HCC cell migration and invasion.

FGD5-AS1 directly targets miR-873-5p in HCC cells

To better understand the mechanism of FGD5-AS1 in regulation of HCC progression, we first analyzed the sub-cellular localization of FGD5-AS1 in Hep G2 and HuH-7 HCC cell lines. Results showed that FGD5-AS1 was mainly located in the cytoplasm (Figure 4A), indicating that this lncRNA potentially functions as a ceRNA by binding to specific miRNAs. By searching the
online bioinformatics database, we predicted that miR-873-5p was a potential downstream target of FGD5-AS1 (Figure 4B). With this in mind, we studied whether FGD5-AS1 bound to miR-873-5p or not. MiR-873-5p mimic was used to achieve miR-873-5p overexpression and the transfection efficiency was validated by the RT-qPCR assay (Figure 4C). Reporter gene assays revealed that transfection of miR-873-5p mimic into HCC cells clearly inhibited the activity of luciferase reporter harboring the FGD5-AS1 sequence with miR-873-5p binding sites. After mutation in the predicted binding site of miR-873-5p within FGD5-AS1, the changes of luciferase activity were nullified (Figure 4D). Furthermore, RNA pull-down assays confirmed that FGD5-AS1 bound to miR-873-5p in HCC cells (Figure 4E). RT-qPCR assays demonstrated that the expression level of miR-873-5p was notably increased due to silencing of FGD5-AS1 in Hep G2 and HuH-7 cells (Figure 4F). Pearson’s correlation analysis suggested that miR-873-5p was negatively correlated with FGD5-AS1 (Figure 4G). RT-qPCR analysis indicated that miR-873-5p was significantly decreased in HCC tissues and cells (Figure 4H-I). This data provides evidence that FGD5-AS1 acted as a sponge of miR-873-5p.

**FGD5-AS1 upregulates GTPBP4 via competitively binding with miR-873-5p**

Using Starbase v2.0 (http://starbase.sysu.edu.cn/), we found that GTPBP4 was a downstream target gene of miR-873-5p and the potential binding sequences were exhibited in Figure 5A. The result of dual luciferase reporter assay revealed that miR-873-5p significantly suppressed only luciferase activity of GTPBP4-WT (Figure 5B). RNA pull-down assay consistently showed that GTPBP4 was only pulled down by bio-miR-873-5p-WT, further validating the interaction of GTPBP4 with miR-873-5p (Figure 5C). RT-qPCR and Western blot analyses demonstrated that over-
expression of miR-873-5p led to the obvious decline of GTPBP4 mRNA and protein levels, confirming that GTPBP4 was a target of miR-873-5p (Figure 5 D,E). We also observed that the miR-873-5p-induced inhibitory effects on the luciferase activity of GTPBP4 were partially retrieved by overexpression of GTPBP4 (Figure 5F).

Our findings certified that depletion of FGD5-AS1 prominently decreased the mRNA and protein expression levels of GTPBP4 (Figure 5 G,H). This data indicates that FGD5-AS1 worked as a ceRNA for miR-873-5p to upregulate GTPBP4.

**The effects of FGD5-AS1/ miR-873-5p/ GTPBP4 axis in HCC**

To further study the effects of FGD5-AS1/miR-873-5p/GTPBP4 axis on the HCC, rescue assays were performed. First, the transfection efficiency in HepG2 cells was confirmed by RT-qPCR (Figure 6A). CCK-8 and EdU assays unveiled that the decreased proliferation of HepG2 cells caused by FGD5-AS1 silence was increased by miR-873-5p inhibitor and subsequently recovered due to knockdown of GTPBP4 (Figure 6 B,C). Flow cytometry analysis of apoptosis showed that the influences of FGD5-AS1 knockdown-induced inhibition on cell apoptosis were canceled out by miR-873-5p inhibitor and the restoration of cell apoptosis occurred when GTPBP4 was silenced (Fig 6D). Wound healing and transwell assays revealed that cell migratory and invasion capacity suppressed by FGD5-AS1 depletion was promoted by miR-873-5p inhibitor and then renewed by repression of GTPBP4 (Figure 6 E,F). Taken together, our results demonstrated that FGD5-AS1 exerted its biological function on HCC cells through miR-873-5p/GTPBP4 axis.

**Discussion**

In this study, we provided direct evidence that FGD5-AS1 acted as an oncogene, and promoted HCC tumor growth and metastasis for the first time. We found that FGD5-AS1 was significantly expressed in HCC tissues and cells. Mechanistic experiments indicated that FGD5-AS1 competitively sponged miR-873-5p to enhance GTPBP4 expression level in HCC cells. These findings suggested that FGD5-AS1 played a direct role in promoting HCC progression, revealing FGD5-AS1 as a novel prognostic marker and therapeutic target in HCC.

LncRNA FGD5-AS1, as novel diagnostic biomarkers, have an intimate connection with the progression of many cancers. For instance, FGD5-AS1 has been reported to promote non-small cell lung cancer cell proliferation through sponging hsa-miR-107 to up-regulate FGFR1 [26]. FGD5-AS1 has also been found to regulate cancer cell proliferation and chemoresistance in gastric cancer through miR-153p/CITED2 axis.27 However, the role of FGD5-AS1 in HCC is completely unknown. We found that the expression of FGD5-AS1 in HCC tissues was significantly higher than that in
the corresponding non-tumor tissues. We believe our study unveiled the carcinogenic effects of FGD5-AS1 in HCC cells. These observations suggest that FGD5-AS1 might be a novel clinical molecular marker for the prognosis of HCC patients. LncRNA FGD5-AS1 has also been reported to promote colorectal cancer cell proliferation, migration, and invasion through upregulating CDCA7 via sponging miR-302c.18 In concert with previous studies, our findings showed that the knockdown of FGD5-AS1 also suppressed malignant phenotypes of HCC cells, including proliferation, migration and invasion, suggesting that FGD5-AS1 functioned as a critical cancer facilitator in the development of HCC.

CeRNA pattern is a very important biological pathway in which lncRNA could participate in the progression of tumors. In our study, FGD5-AS1 was found to be expressed principally in the cytoplasm of HCC cells. Hence, we hypothesized that FGD5-AS1 might serve as a ceRNA in HCC and then searched for potential interactions with miRNAs. Then, we performed bioinformatics analysis and found that miR-873-5p possessed complementary sequences with FGD5-AS1. Previous studies reported that miR-873-5p functioned as a tumor suppressor in various cancers.28-30 Thus, we speculated that FGD5-AS1 exerted oncogenic effects on HCC cells via interacting with miR-873-5p. Our results demonstrated that FGD5-AS1 negatively regulated miR-873-5p. Furthermore, bioinformatics analysis suggested that GTPBP4 might be a downstream target of miR-873-5p. In recent studies, GTPBP4 has been reported to be a tumor promoter in several cancers including HCC.23,31,32 However, the relationship between miR-873-5p and GTPBP4 in HCC remains unknown. In this study, we used luciferase reporter assay and RNA pull down to justify that GTPBP4 was a direct target of miR-873-5p. Additionally, the results of rescue assays revealed that FGD5-AS1 promoted the tumorigenesis and metastasis of HCC through miR-873-5p/GTPBP4 axis. However, the impaired effect after HCC cells co-transfected with miR-873-5p and GTPBP4, the abundance of FGD5-AS1 compared to miR-873-5p in HCC cell lines and more in vivo experiments were needed to further confirm our results.

In summary, FGD5-AS1 is the host gene for the miR-3127-5p, therefore, it induces gene silencing by binding to target sites within the 3′UTR of the targeted GTPBP4; second, EGFR-AS1 functions as a ceRNA and bases its activity on a sequence-specific interaction with miRNA, thus reducing their action on mRNA targets by titrating the amount of free miRNA.

Our study has provided evidence that lncRNA FGD5-AS1 hold an essential role in the progression of HCC. More importantly, the discovery of the FGD5-AS1/miR-873-5p/GTPBP4 axis offered new insights into the molecular basis of liver cancer and a novel prospect for the development of new diagnostic and therapeutic strategies.

Figure 6. The effects of FGD5-AS1/ miR-873-5p/GTPBP4 axis in HCC. A) The expression levels of GTPBP4 and miR-873-5p in Hep G2 cells transfected with sh-GTPBP4 or miR-873-5p inhibitor; *p<0.05, **p<0.01 vs sh-NC inhibitor group. B,C) CCK-8 and EdU assays were performed to evaluate the cell viability. D) Flow cytometry analysis was performed to measure the cell apoptosis. E,F) Wound healing and transwell assays were performed to determine the migration and invasive capability of cells. All data was presented as mean ± SD and each assay was conducted in triplicate; *p<0.05, **p<0.01 vs sh-NC inhibitor group; #p<0.05, ##p<0.01 vs sh-FDG5-AS1+NC inhibitor group; 100x magnification; scale bar: 50 μm.
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