miR-125b suppresses cellular proliferation by targeting c-FLIP in gallbladder carcinoma

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Abstract. Gallbladder carcinoma (GBC) is the most common malignant tumor of the biliary tract. The incidence rate of gallbladder cancer ranks sixth among gastrointestinal types of cancer, and its incidence is increasing each year. Further clarification of the pathogenesis of GBC is essential, and identification of novel effective treatments is required. It has been previously demonstrated that high expression of the anti-apoptotic protein cellular Fas-associated death domain-like interleukin-1-converting enzyme inhibitory protein (c-FLIP) in GBC inhibited apoptosis in gallbladder cancer cells. In subsequent experiments, it was observed that microRNA (miR)-125b could target c-FLIP and inhibit the protein expression of c-FLIP by binding to the 3'untranslated regions of c-FLIP mRNA. In addition, the expression of miR-125b in GBC was significantly decreased, and the growth of gallbladder cancer cells was inhibited by the overexpression of miR-125b. The present study demonstrated that miR-125b could suppress the proliferation of gallbladder cancer cells by targeting c-FLIP. c-FLIP enriched the target gene pathway of miR-125b and may serve as a novel target for the treatment of GBC.

Introduction

Gallbladder carcinoma (GBC) is the most common malignancy of the bile duct, with its incidence ranking sixth in gastrointestinal tumors, and its prevalence is increasing annually (1). GBC is characterized by its high malignancy, and metastasis can occur in the early stages of disease. Traditional therapeutics, including surgery and chemoradiotherapy have a poor effect on GBC, and the 5-year survival rate is currently >5% (1,2). Therefore, investigating the pathogenesis and identifying effective treatments for GBC is an important area of research.

Receptor-mediated apoptosis serves an important role in killing tumor cells (3). The induction of apoptosis primarily occurs through a combination of tumor necrosis factor superfamily members, including tumor necrosis factor α (TNF-α), factor associated suicide legend (FAS-L) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and their receptors on the cell membrane, therefore, activating proteins involved in caspase activity, which then hydrolyse and destroy the cellular structure and proteins (3). However, tumor cells often exhibit anti-apoptotic capabilities, and the existence of cellular Fas-associated death domain-like interleukin-1-converting enzyme inhibitory protein (c-FLIP/CFLAR) results in the insensitivity and tolerance of tumor cells towards TNF-α, Fas-L and TRAIL (4-7). The overexpression of c-FLIP inhibits receptor-mediated apoptosis and promotes the growth of tumors (5). In colon cancer, prostate cancer, pancreatic cancer and melanoma, c-FLIP has been identified to be highly expressed, and patients expressing high levels of c-FLIP had a poor prognosis (7). Our previous research also confirmed that c-FLIP was highly expressed in GBC (8). The knockdown of c-FLIP has been hypothesized to significantly enhance TRAIL-induced apoptosis in gallbladder cancer cells (8), and the results of a previous study indicated that c-FLIP has become a novel target for tumor therapy (9). However, the regulation of the expression of c-FLIP in tumors and its associated mechanism of action has not been completely elucidated.

The mutations or heterotopic expressions of microRNAs (miRs) are associated with multiple types of human cancer and may serve an important role in the development of a number of different types of cancer, including breast cancer, hepatocellular carcinoma and lung cancer (10). Aberrant expression of various miRs has been identified in a variety of tumors, and are associated with certain clinical features of tumors, including drug resistance and poor survival (10,11). It has been reported that miR-512-3p can inhibit the expression of c-FLIP in HepG2 hepatocellular carcinoma cells in combination with the 3'untranslated regions (3'UTR) of c-FLIP (12). Therefore,
the present study investigated whether the regulation of c-FLIP by miR could affect the function of tumor cells and the development of GBC.

Our previous research demonstrated that the c-FLIP protein was significantly upregulated in GBC tissues (as shown by immunohistochemistry), and inhibiting the expression of c-FLIP could significantly enhance the effect of TRAIL-induced apoptosis on gallbladder cancer cells (8). miRs that regulated the expression of c-FLIP were subsequently predicted through bioinformatic analysis, and a microarray to detect microRNA expression profile in GBC and normal gallbladder tissue samples were further utilized. The results of the present study demonstrated that miR-125b could significantly inhibit the growth and colony-forming ability of gallbladder cancer cells, and miR-125b was observed to be significantly downregulated in gallbladder cancer tissues. The regulatory effect of miR-125b on c-FLIP 3'UTR was verified by a luciferase reporter gene experiment. This confirmed that the low expression of miR-125b in GBC increased the RNA expression of c-FLIP and promoted the growth of GBC cells. Taken together, these results suggest that c-FLIP may be a novel target of miR-125b. In addition, miR-125b may be used as a candidate target for the clinical treatment of GBC.

Materials and methods

Human tissues sample. In the present study, a total of 23 patients with GBC (including 13 males and 10 females) were enrolled between April 2015 and October 2016 at Huashan Hospital (Fudan University, Shanghai, China). The median age of the patients was 60 years (range, 32-85) at the time of surgery. The tumor and adjacent normal tissues were obtained from patients following resection from the primary neoplasms. The clinical features of patients are presented in Table I. Tissues were graded using the Tumor‑Node‑Metastasis grading system as described previously (8). The present study was approved by the Ethics Committee of Huashan Hospital, and written informed consent was obtained from all patients.

Cell culture and transfection. The gallbladder cancer cell lines, SGC-996 and GBC-SD, were used in the present study. SGC-996 cell line was provided by the Academy of Life Sciences, Tongji University (Shanghai, China), and the GBC-SD cells were purchased from the Shanghai Institute for Biological Sciences (Shanghai, China). SGC-996 and GBC-SD cells were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37˚C.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Fresh tissues of unknown thickness were rinsed with saline water and ground with liquid nitrogen. Total RNA was isolated from GBC tissues, adjacent tissues and both cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For reverse transcription of total RNA containing miRNAs, the miScript PCR system (Qiagen, Inc.) was used and the reverse transcription temperature protocol was 37˚C for 60 min and 95˚C for 5 min. The miScript II RT kit (Qiagen, Inc.) is part of the miScript PCR system for miRNA detection and quantification. cDNA generated with the miScript II RT kit was used as a template for real-time PCR, with the expression quantified by PCR with specific primers and probes using TaqMan microRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The thermocycling conditions were: 90˚C For 20 sec; followed by 40 cycles of 95˚C for 1 sec and 60˚C for 20 sec. The primer sequences used for qPCR were the same as those in two previous studies (13,14). The primer sequences used for qPCR were as follows: miR-125b forward, 5'-GCT CCC TGA GAC TTA C-3' and reverse, 5'-CATGCAGGTGTCGCCAGGT-3'; and U6 forward, 5'-GCTTCGGACCCAGATCTA-3' and reverse, 5'-GCGAGCACAGAATATACGAC-3'. Relative expression was quantified using the 2^(-DeltaCq) method (8).

Western blot analysis. miR-125b overexpression or control-treated GBC-SD and SGC-996 cells were collected and lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing proteinase inhibitor cocktail (cat. no. P8340; Sigma-Aldrich; Merck KGaA). The protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 60 µg of protein was separated by SDS-PAGE (12% gels) and transferred onto polyvinylidene difluoride membranes. The membranes were subsequently blocked in 5% fat-free milk at room temperature for 2 h and incubated with anti-c-Flip (cat. no. ab8421; 1:1,000; rabbit polyclonal, Abcam) and anti-GAPDH (cat. no. sc-32233; 1:1,000; mouse monoclonal; Santa Cruz Biotechnology, Inc.) primary antibodies overnight at 4˚C. Following incubation with goat anti-mouse (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) or goat anti-rabbit IgG-HRP (cat. no. sc-2005; 1:5,000; Santa Cruz Biotechnology, Inc.) secondary antibodies at room temperature for 2 h. The membranes were visualized using the...
enhanced chemiluminescence-Plus kit (GE Healthcare Life Sciences), according to the manufacturer's protocols.

**Plasmid construction and luciferase reporter experiment.** In order to determine miRNA targeting of the 3'-UTR region of c-FLIP, the full length of the c-FLIP 3'-UTR was amplified and cloned into a pmiR-REPORT luciferase vector (Promega Corporation). DNA was extracted from the tissue samples using the PicoPure™ DNA Extraction kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols, and was treated with DNA polymerase (Promega Corporation). The temperature protocol was as follows: 95°C for 3 min, followed by 30 cycles of 94°C for 40 sec, 56°C for 35 sec and final extension at 72°C for 60 sec. QuickMutation™ Site-Directed Mutagenesis kit (Beyotime Institute of Biotechnology) was then used to generate the mutated vector by replacing the miR-9-5p binding site nucleotides, according to the manufacturer's protocol.

The cells were inoculated into 24-well plates, which were transfected with the expression plasmids. Cells were collected at 24-48 h following transfection, and were washed with PBS. Subsequently, 80 µl 1x Passive Lysis Buffer (Promega Corporation) was added to each well, and placed on a shaker at room temperature for 120 min. The cell lysates (8-10 µl/well) were used for detection. The program was set with a 10-sec pre-read delay, followed by a 30-sec measurement period. The supernatant was then added to the plate, and the firefly luciferase reaction substrates and Stop & Glo® reagents (Promega Corporation) were added, including 30 µl of both LARII and Stop & Glo® reagent/well. Firefly luciferase reaction substrates and Stop & Glo® reagents were added to the fluorometer tube, and the relative activity was measured.

**Colonies formation assay.** The cells in the logarithmic growth phase following transfection were detached using trypsin to form a single-cell suspension, and inoculated into 24-well plates with 500 cells/well, and cultured for 1-2 weeks at 37°C. When visible colonies appeared, the culture was terminated. Following washing with PBS twice, cells were treated with 4% paraformaldehyde for 15 min at 37°C. Cells were subsequently stained with 0.25% crystal violet dye for 10-30 min at 37°C, and the residual crystal violet was removed with water slowly, and the cultures were air-dried. The number of colonies were counted in five randomly selected fields using a light microscope at a x4 magnification. The colony numbers were calculated and analyzed statistically.

**Lentivirus production and transduction.** The primary miRNA sequence was amplified from normal genomic DNA and the open reading frame of c-Flip was amplified from cDNA. Using these amplified sequences, the green fluorescent protein fragment of the pWPXL mock vector was replaced to form the lentivirus expression vector pWPXL-miR-125b and pWPXL-c-FLIP. After transfecting pWPXL-miR-125b, pWPXL-c-FLIP or pWPXL-mock with the envelope plasmid pMDG2 and the packaging plasmid psPAX2 into 293T cells using Lipofectamine 2000 at 48 h, the virus particles were subsequently harvested, and GBC-SD and SGC-996 cells were infected with the recombinant lentivirus-transducing units and 6 µg/ml polybrene.

<table>
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<tr>
<th>Variables (n=12)</th>
<th>miR-125b expression</th>
<th>Total (n=23)</th>
<th>P-value</th>
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miR, microRNA; TNM, Tumor-Node-Metastasis.

Figure 1. Expression of miR-125b in gallbladder carcinoma is significantly downregulated. The expression of miR-125b-5p in 23 pairs of gallbladder carcinoma and adjacent tissues were detected, and U6 was used as a control. Statistical analysis was performed using the Wilcoxon matched-paired rank test. miR, microRNA.

**Bioinformatics analysis.** TargetScan (targetscan.org/vert_72/) was used to predict miRNAs which regulated c-Flip.

**Statistical analysis.** The results of the present study were analyzed using the STATA 8.0 statistical software (StataCorp LP). Differences among groups were analyzed using Wilcoxon signed-rank test, a one-way ANOVA with Bonferroni post hoc test, or a Student's t-test. The association between miRNA expression and the clinicopathological variables of the patients was analyzed using the χ² test or Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Expression of miR-125b in GBC tissues is lower compared with the normal tissues. In order to identify miRs that regulated the expression of c-FLIP, a bioinformatics analysis was
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performed and it was identified that a series of miRs could target the c-FLIP gene, including miR-125b, miR-93, miR-10a, miR-20a, miR-20b, miR-143, miR-504, miR-150 and miR-149. The RT-qPCR results demonstrated that the expression of miR-125b was significantly decreased in GBC tissues when compared with 23 cases of matched normal gallbladder tissues (Fig. 1). This suggests that miR-125b functioned as a tumor suppressor gene in GBC. We hypothesized that miR-125b may inhibit the expression of c-FLIP. Therefore, the present study investigated whether miR-125b expression was associated with the clinicopathological features of patients with GBC. As presented in Table I, the statistical analysis demonstrated that miR-125b expression was significantly associated with the grade of GBC (P=0.0361).

miR-125b inhibits the proliferation of gallbladder cancer cells. To examine the effects of miR-125b on the cell growth of gallbladder cancer cells, the present study transfected gallbladder cancer cells with miR-125b analogs (miR-125 mimic), and detected the cellular proliferation rate of the gallbladder cancer cells GBC-SD and SGC-966. The RT-qPCR results demonstrated that miR-125b was efficiently overexpressed in gallbladder cancer cells (Fig. S1A and B). miR-125b was observed to have an inhibitory effect on the proliferation of gallbladder cancer cells (Fig. 2A and B). This indicated that increased expression of miR-125b in GBC may reduce or delay cellular proliferation and tumor growth.

miR-125b weakens the colony formation ability of gallbladder cancer cells. A colony formation assay was subsequently performed in order to examine the influence of miR-125b on the colony forming ability of gallbladder cancer cells. miR-125b was overexpressed in the gallbladder cancer cell lines GBC-SD and SGC-966, and the number of colonies formed was significantly decreased (Fig. 3A and B). The cell activity analysis and the alteration of the colony formation ability of gallbladder cancer cells suggested that miR-125b mimics significantly decrease the colony formation ability of gallbladder cancer cells. Therefore, these results suggest that miR-125b functions a potential tumor suppressor and serves an important role in inhibiting the initiation and development of GBC.

miR-125b suppresses the expression of c-FLIP by interacting with the 3' UTR of the c-FLIP mRNA. It has been previously reported that the overexpression of c-FLIP could inhibit the apoptosis of gallbladder cancer cells (8). In order to determine whether miR-125b inhibited the growth of gallbladder cancer cells by regulating the expression of c-FLIP, the present study initially predicted the binding sites of miR-125b and the 3'UTR of c-FLIP mRNA (Fig. 4A). By cloning the c-FLIP mRNA 3'UTR and c-FLIP mRNA 3' UTR mutation into the luciferase report plasmid vector, the present study examined the change of luciferase activity following the overexpression of miR-125b, and found that miR-125b inhibited the luciferase activity. In addition, this inhibitory effect was not observed when the binding sites were mutated (Fig. 4B). Furthermore, the overexpression of miR-125b significantly inhibited the protein expression of c-FLIP (Fig. 4C). This indicated that miR-125b may inhibit the expression of c-FLIP through direct binding with the 3'UTR of c-FLIP mRNA, and further demonstrated that miR-125b may suppress the proliferation of gallbladder cancer cells through reducing the expression of c-FLIP.

Discussion

The treatment of GBC is challenging for hepatobiliary surgeons in a clinical setting; however, the combination of surgery with chemotherapy and radiotherapy serves as the principal means of treatment for patients with GBC (15,16). As the majority of tumors are at an advanced stage of the disease at the time of diagnosis, only 10-30% of patients with GBC can undergo radical surgery (15-17). The mutation rate of the P53 gene in GBC is high (18-20) and the multidrug-resistance gene MDR1 has also been demonstrated to be expressed in GBC (21), therefore, contributing to the insensitivity of GBC to radiotherapy and chemotherapy. Previous studies have reported that the response rate of gemcitabine and platinum compounds and
5-FU, when used alone or in combination was >30% (22-24). The overall treatment efficacy of GBC is poor, with 5-year overall survival rate of only 2-5% (1,2). In addition to early detection and surgical treatment, it is imperative to identify novel and more effective methods for the treatment of GBC.

c-FLIP is an inhibitory protein of cellular apoptosis, which was initially reported by Irmler et al (25). c-FLIP is an analog of procaspase-8 and procaspase-10, which can bind to FADD, interfering with the recruitment of caspase-8/caspase-10, thereby blocking the conduction of death receptor signals and inhibiting apoptosis (26). c-FLIP contains two sequential terminal domains, which are termed the death effector domains (5). In c-FLIP, there is an extending domain from the C-terminal that contains a caspase-like domain; however, the two amino acid residues, Cys and His, in this caspase-like domain are replaced by Tyr, which results in the proteolytic
enzyme activity of c-FLIP being lost (27). At present, the high expression of c-FLIP has been detected in various tumors, including colon cancer, gastric cancer, pancreatic cancer and melanoma. In colon cancer, the expression level of c-FLIP is one of the independent factors that affect the prognosis of patients (4). The high expression of c-FLIP has an antagonistic effect in anti-androgen receptor drugs in patients with prostate cancer that are ineffective in the treatment of castration (28). Further studies have confirmed that inhibiting the expression of c-FLIP in melanoma, and in prostate and liver cancer can enhance the apoptosis induced by a tumor in TRAIL and CD95L (29-31). In addition, the sensitivity of tumor cells to chemotherapeutic drugs, including taxol, cisplatin and 5-FU is elevated in tumors such as colon and cervical cancer (32,33). The results of the present study indicated that c-FLIP may function as an oncogene and its high expression may be associated with tumor growth. The present study demonstrated that c-FLIP is highly expressed in GBC, and may suppress cellular apoptosis through the regulation of TRAIL.

miR is a type of single-stranded non-coding RNA of ~19-25 nucleotides in length. It can bind to the mRNA coding region or UTR, which directly results in the degradation of target genes or inhibits the translation process of target genes, thereby influencing their expression at the post-transcriptional level (34,35). We have previously demonstrated that a series of miRNAs could target the c-FLIP gene, including miR-125b, miR-93, miR-10a, miR-20a, miR-20b, miR-143, miR-504, miR-150, miR-149 and miR-125b was associated with the occurrence, development, metastasis and prognosis of human tumors (36). Shi et al (37) revealed that miR-125b inhibited the proliferation of glioma stem cells by blocking the cell cycle at the G1/S phase. A previous study also demonstrated that miR-125b suppressed the growth of hepatocellular carcinoma cells by inhibiting the phosphorylation of Akt and the cell cycle, additionally, the survival rate was higher in patients with cancer expressing elevated levels of miR-125b (38). It has also been reported that miR-125b could attenuate the growth of melanoma by directly regulating the expression of the c-Jun protein (39). Studies have reported that miR-125b is an important prognostic indicator in colon cancer, and patients with a high expression of miR-125b had a larger tumor volume, exhibited high tumor invasion and had a poor prognosis (40,41). Furthermore, miR-125a and miR-125b could act on tumor necrosis factor α-induced protein 3, and promote the activation of the NF-κB pathway in diffuse large B-cell lymphoma (42). In conclusion, the results of these studies suggest that miR-125b serves an important role in a variety of tumors. We hypothesize that miR-125b could target c-FLIP in GBC; however, to the best of our knowledge, the regulation of miR-125b on c-FLIP has not yet been investigated.

Using bioinformatics analysis, the present study demonstrated that miR-125b can bind to the 3'UTR of c-FLIP mRNA. Furthermore, the use of a luciferase reporter gene assay confirmed that miR-125b can inhibit the expression of c-FLIP, therefore, significantly inhibiting the protein expression of c-FLIP. In addition, it was observed that miR-125b was lowly expressed in GBC tissues, and the overexpression of miR-125b could increase the proliferation and colony formation capacity of gallbladder cancer cells. This suggests that miR-125b may function as a potential tumor suppressor. However, whether miR-125b may also inhibit tumor cell growth in other tumors remains to be further studied. The results of the present study revealed the important role of the miR-125b-c-FLIP signal in the growth of gallbladder cancer cells, providing a novel strategy for clinical treatment of GBC.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
XY and GW conceived the study, carried out the experimental design and data interpretation, and prepared and revised the manuscript. HJZ and HDZ performed the experiments. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Huashan Hospital (Fudan University) and written informed consent was obtained from all patients.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


