Down-regulation of FoxM1 inhibits viability and invasion of gallbladder carcinoma cells, partially dependent on induction of cellular senescence

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Abstract

AIM: To investigate the effect of knockdown of fork-head box M1 (FoxM1) on the proliferation and invasion capacities of human gallbladder carcinoma (GBC)-SD cells.

METHODS: Four FoxM1 shRNAs were transfected into GBC-SD cells with Lipofectamine 2000 to select the appropriate shRNA for down-regulation of FoxM1. A recombinant lentivirus for shFoxM1 (Lv-shFoxM1), which expresses FoxM1-specific shRNA, and a negative control carrying green fluorescent protein, which expresses a scrambled RNA, were constructed. After transfection with the recombinant adenovirus and screened with puromycin, RT-PCR and Western blot were utilized to evaluate the inhibition efficiency. Cell viability was evaluated by MTT assay, and cell migration and invasion were assessed using the Transwell system. Cells were suspended in serum-free medium and seeded into Transwell inserts either uncoated (for migration assay) or coated (for invasion assay) with growth factor-reduced Matrigel. To verify the involvement of FoxM1 in the senescence of tumor cells, staining of senescence β-galactosidase (SA β-gal), the widely used biomarker of cellular senescence, was also performed.

RESULTS: After successful transfection of four FoxM1 small interfering RNAs (shRNAs) with Lipofectamine 2000, the shF1822 was selected as the most appropriate shRNA according to its obvious inhibitory effect. The recombinant adenovirus was then constructed with the shF1822 and successfully transfected into the GBC-SD cells, resulting in the significant inhibition of FoxM1 expression at both the mRNA and protein levels, compared with the negative control (P < 0.05). After transfection, down-regulation of FoxM1 significantly inhibited cell viability according to the MTT assay (P < 0.05). In addition, Transwell migration and invasion assays also suggested the suppression of invasion ability of the transfected cells. SA β-gal staining showed that down-
regulation of FoxM1 could induce more senescent GBC cells (P < 0.05), suggesting the possible involvement of the senescence process of the FoxM1-deficient cells in GBC.

CONCLUSION: FoxM1 is functionally involved in viability of GBC cells, partially dependent on the induction of cellular senescence, and is a potential target for GBC therapy.

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Key words: Forkhead box M1; Gallbladder carcinoma; Senescence; Viability; Invasion

Core tip: Gallbladder cancer is characterized by early metastases, thus it is in urgent need to identify novel therapies to enhance the therapeutic effect. We have previously reported that Forkhead box M1 (FoxM1) expression was closely correlated with gallbladder carcinoma differentiation, Nevin stage and metastasis, indicating the potential roles of FoxM1 in gallbladder carcinoma. In this study, by regulating the expression of FoxM1 with small interfering RNAs, we demonstrated the impact of FoxM1 on cellular viability in a human gallbladder carcinoma cell line, which was probably via the regulation of cellular senescence, revealing FoxM1 as a potential target for gallbladder carcinoma therapy.

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INTRODUCTION

Gallbladder carcinoma (GBC) is the fifth most common malignant tumor of the digestive system, and the most common malignant neoplasm of the biliary tract, with an incidence of 1-2 cases/100000 worldwide. However, in Eastern countries such as China and South America, this neoplasm is more common, with an incidence up to 96 cases/100000[1,2]. GBC is characterized by early lymph node and distant metastases, thus only 10% of patients present with early-stage disease and are candidates for surgical resection[3]. Currently, the prognosis of advanced GBC is very poor, as the overall survival is less than 1 year following diagnosis[4]. As no specific chemotherapy or radiotherapy for the disease has emerged with satisfying effects, many researchers have been trying to explore new approaches to benefit GBC patients, but only achieved limited progress[5-8]. It is then in urgent need to identify novel therapies to enhance the therapeutic effect and improve the survival of GBC patients.

Forkhead box M1 (FoxM1), which is characterized by the forkhead box domain, is a proliferation-associated transcription factor that has important roles in cellular proliferation, cell cycle progression, tissue repair and carcinogenesis[9,10]. We have previously reported the involvement of FoxM1 in cellular senescence in hepatocellular carcinoma (HCC) through p53-FoxM1 pathway, indicating a new promising target for treating digestive cancers[11,12]. In addition, our previous study also showed that FoxM1 expression was closely correlated with GBC differentiation, Nevin stage and metastasis, and that GBC patients with highly expressed FoxM1 would have a poorer overall survival by multivariate analysis, indicating the potential roles of FoxM1 in GBC[13]. Thus in this study, by regulating the expression of FoxM1 with small interfering RNAs (siRNAs), we explored the impact of FoxM1 on cellular viability in a human GBC cell line, which was probably via the regulation of cellular senescence.
construct was isolated using plaque screening, purification and amplification. Protocol of lentivirus infection was according to the GenePharma Recombinant Lentivirus Operation Manual (http://www.genepharma.com).

**Reverse transcription-polymerase chain reaction analysis**

Total RNA was isolated from cell groups using the RNAfast200 Total RNA Extract Kit (Fastgene, Shanghai, China). Primers used in PCR were designed according to the reported FoxM1 cDNA sequence. The primer sequences were 5'-CAC CCC AGT GCC AAC CGC TAC TTG-3' (forward), and 5'-AAA GAG GAG CTA TCC CCT CCT CAG-3' (reverse). The primer sequences of β-actin were 5'-CGC GAG AAG ATG ACC CAG AT-3' (forward) and 5'-GCA CTG TGT TGG CGT ACA GG-3' (reverse). PCR cycling parameters included an initial denaturation step at 95 ℃ for 5 min; 35 cycles at 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 1 min; and a final elongation phase at 72 ℃ for 7 min. Each sample was assayed in triplicate.

**Western blot**

Cells were lysed in ice-cold RIPA lysis buffer (Beyotime Inc., NanTong, China). The total protein concentration was determined with the Bradford reagent (Beyotime Inc.). Equivalent amounts of proteins were then separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, United States). The membranes were subsequently immunoblotted with the appropriate primary antibody at 4 ℃ for 12 h, and then incubated with HRP conjugated anti-goat or anti-rabbit antibody (Santa Cruz, CA, United States). Signals were detected on X-ray films using the ECL detection system (Pierce, Rockford, IL, United States). Equal protein loading was assessed by the expression of β-actin.

**MTT assay for cell viability**

GBC-SD cells were seeded at 5 × 10^3 cells per well in 96-well flat-bottom plates before transfection. For the assay, 20 μL of MTT solution (5 g/L) was added to each well, and the cells were incubated for 4 h. Then supernatants were removed and formazan crystals were dissolved in 200 mL dimethylsulfoxide. After the insoluble crystals were completely dissolved, the absorbance values at 490 nm were measured using a microplate reader (Bio-Rad, Hercules, CA, United States).

**Senescence β-galactosidase assay**

Senescence β-galactosidase (SA-β-gal) staining is widely used to assess cellular senescence in vitro and in vitro, with the positive green or blue-colored staining of β-galactosidase at pH 6.0 being remarkably increased in senescent cells. Senescent cells in the GBC-SD cell line were analyzed using a SA-β-gal staining kit (Beyotime Inc., Nantong, China) according to the manufacturer’s instructions. The percentage of SA-β-gal positive cells was calculated by counting the cells in five random fields (at least 100 cells) using bright-field microscopy. The staining results were recorded as “positive” or “negative”, according to the method reported by ten Poel et al.[14].

**Cell invasion and migration assays**

Cell invasion and migration assays were performed using Transwell permeable supports with 8 μm pore size (Costar, Cambridge, MA, United States). Cells were suspended in serum-free medium and seeded into Transwell inserts either uncoated (for migration assay) or coated (for invasion assay) with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA). Bottom wells were filled with complete medium, and after 24 h, the invaded cells were fixed with methanol and stained with a crystal violet solution. The number of cells that penetrated the membrane was determined by counting the mean cell number in five randomly selected high-power fields.

**Statistical analysis**

All data are expressed as mean ± SD. To compare the means of normally distributed variables, analysis of variance or Student’s t test was applied. A P value less than 0.05 was considered significant in all tests. All analyses were performed using the SPSS software version 19.0 (SPSS Inc, Chicago, IL, United States).

**RESULTS**

**Expression of FoxM1 is significantly correlated with the prognosis of GBC patients**

The expression of FoxM1 has been reported to be associated with the prognosis of cancer patients in a series of tumors[15-17]. Likewise, our previous study has also demonstrated the potential impact of FoxM1 on the poor prognosis of GBC patients[18]. As shown in Figure 1A-F, the expression of FoxM1 in GBC tissues was significantly higher than that in the pericarcinoma tissues and adjacent non-GBC gallbladder samples (P < 0.05). Furthermore, the Kaplan-Meier analysis also demonstrated that GBC patients with positive FoxM1 expression had a poorer overall survival than those with negative FoxM1 expression (P < 0.05) (Figure 1G).[13]

**Analysis of inhibitory efficiency of the shRNAs**

To investigate the inhibitory efficiency of the shRNAs in GBC-SD cells, PCR and Western blot were performed 48 h after the shRNA transfection. We found that the FoxM1-homo-1822 was more efficient and could significantly inhibit the expression of FoxM1 (Figure 2). Thus, the FoxM1-homo-1822 was selected as the optimal shRNA of FoxM1 in the following experiments.

**Silencing of FoxM1 by adenovirus transfection at the mRNA and protein levels**

GBC-SD cells, which have a high level of FoxM1 expression, were stably transfected with the FoxM1 shRNA or negative control (NC-shRNA) (Figure 3). After 3-wk
Figure 1  Immunohistochemical analysis of Forkhead box M1 in gallbladder carcinoma (A and D), pericarcinoma (B and E) and healthy tissues (C and F). Typically, immunohistologic features showed high levels of Forkhead box M1 (FoxM1) expression in carcinoma, low levels of FoxM1 expression in pericarcinoma, and negative staining of FoxM1 in healthy gallbladder tissues. G: Kaplan-Meier analysis of the GBC patients, indicating the poorer survival of the patients with positive FoxM1 expression ($P < 0.05$). Original magnification (× 100, top; × 400, bottom).

Figure 2  Expression of Forkhead box M1 in gallbladder carcinoma-SD cells transfected with different shRNAs, detected by RT-PCR (A) and Western blot (B). Results showed that the shF1822 could significantly inhibit the expression of Forkhead box M1 (FoxM1), both at the mRNA and protein levels. Data are from three independent experiments.
screening with puromycin, the total RNA and protein of the transfected cells were isolated and analyzed by RT-PCR and Western blot, respectively. Compared with the blank (no siRNA) and NC-shRNA transfected cells, the expression of FoxM1 was significantly suppressed in cells transfected with FoxM1 shRNA, at both the mRNA and protein levels ($P < 0.05$) (Figure 4).

Defective viability induced by FoxM1 down-regulation

As shown in Figure 5, down-regulation of FoxM1 expression in GBC-SD cells caused significant inhibition of cell viability compared with the control and NC-shRNA cells ($P < 0.05$). Furthermore, the Transwell system was utilized to determine whether down-regulation of FoxM1 affected the invasive and migratory ability of GBC-SD cells. Compared with the control and NC-shRNA cells, suppression of FoxM1 significantly inhibited the invasion and migration of GBC-SD cells, as indicated by a marked decrease in cell invasion and migration.

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**Figure 3** Representative photograph (× 100) showing recombinant adenovirus transfection efficiency evaluated by fluorescence microscopy (transfected with the negative control, top; transfected with the shF1822, bottom). A, D: Under an ordinary light microscope; B, E: Under a fluorescence microscope; C, F: Superimposed image of the two images.

**Figure 4** Expression of Forkhead box M1 in gallbladder carcinoma-SD cells transfected with the recombinant adenovirus with shF1822, detected by RT-PCR (A) and Western blot (B). Results showed that the shF1822 could significantly inhibit the expression of Forkhead box M1 (FoxM1), both at the mRNA and protein levels. Data are from three independent experiments.

**Figure 5** Down-regulation of Forkhead box M1 inhibited cell viability as revealed by MTT assay. Compared with the control and NC groups, shF1822 significantly inhibited the proliferation of GBC-SD cells at 48 h and 72 h. Data are mean ± SD from three independent experiments. *$P < 0.05$ vs control.
Figure 6  Down-regulation of Forkhead box M1 suppressed cell invasion and migration in gallbladder carcinoma-SD cells as measured by the Transwell cell coated or uncoated with growth factor-reduced Matrigel. Compared with the control group (A) and NC group (B), shF1822 (C) significantly inhibited the invasion ability of gallbladder carcinoma (GBC)-SD cells; D: Cells that invaded the bottom well were counted from 10 different visual fields. Compared with the control group (E) and NC group (F), shF1822 (G) significantly inhibited the migration ability of GBC-SD cells; H: Cells that invaded the bottom well were counted from 10 different visual fields. Data are mean ± SD from three independent experiments. *P < 0.05 vs control.
decrease in the number of cells that invaded the bottom well (P < 0.05, Figure 6).

**Senescence of GC cells induced by FoxM1 down-regulation**

We further examined the mechanism behind FoxM1 depletion-mediated impaired viability in GBC-SD cells. As a significant molecule for regulating cellular senescence in a series of malignancies, FoxM1 depletion might also trigger a cellular senescence program in GBC. SA-β-gal staining was thus performed\(^{(18)}\). Positive SA-β-gal staining was observed in approximately 20% of GBC-SD cells treated with FoxM1 siRNA, which was significantly higher than those in the control group and NC-shRNA group (P < 0.05, Figure 7).

**DISCUSSION**

In the previous and present study, we demonstrated the overexpression of FoxM1 in both GBC-SD cells and primary GBC tissues. FoxM1, a transcription factor ubiquitously expressed in most cancer cells, is essential for sustaining proliferation of GBC-SD cells. We have previously demonstrated the correlation between high FoxM1 expression and the poor prognosis of GBC patients, and in the present study, the impaired viability and invasion ability of GBC-SD cells were also induced by down-regulation of FoxM1. Furthermore, SA-β-gal staining indicated that FoxM1 down-regulation could induce the senescence of GBC-SC cells, suggesting that the impaired viability of GBC-SC cells was probably partially dependent on the induction of cellular senescence.

FoxM1, a critical regulator of cell cycle progression, has important roles in cell proliferation, organogenesis, senescence and carcinogenesis\(^{(9,10)}\). Genome-wide gene expression profiling of cancers has independently identified FoxM1 as one of the genes whose expression is most commonly up-regulated in human solid tumors, such as liver, prostate, brain, breast, lung, colon, and pancreatic tumors\(^{(19)}\). In addition, subsequent studies also demonstrated that high expression of FoxM1 predicted the poor prognosis of several malignancies, such as gastric cancer, lung cancer, and liver cancer\(^{(15,16,20)}\). Likewise, we discovered that the FoxM1 was also up-regulated in GBC. Furthermore, according to our previous results, we demonstrated that high expression of FoxM1 was closely correlated with differentiation, Nevin stage and metastasis of GBC, and thus the prognosis of GBC patients\(^{(13)}\).

Consistent with the observed decreased cell proliferation, FoxM1 deficiency has been reported to be associated with reduced expression of many cell cycle-regulatory genes that are known to potentely promote progression through the S-, G2- and M-phases of the cell cycle. Thus, knockdown of FoxM1 was supposed to be associated with impaired proliferation of tumor cells. Previous studies have also verified the inhibition of viability in FoxM1-deficient cells in a series of tumor cells, indicating the possible similar effect of FoxM1 on GBC cells\(^{(12,21,22)}\). In
our study, consistent with the supposition, we found that down-regulation of FoxM1 by shRNA transfection could significantly inhibit the viability of GBC-SD cells. In addition, Transwell assays also demonstrated suppressed migration and invasion ability of the GBC-SC cells transfected with the FoxM1 shRNA, further verifying the impaired proliferation of FoxM1-deficient cells.

Cellular senescence is an irreversible growth arrest, characterized by decreased cell proliferation, combined with accumulation of mitotic defects and chromosomal instability[24]. This permanent exit from the cell cycle represents an important tumor suppression mechanism. In accordance with its proliferation stimulating function, FoxM1 prevents both oncogene- and oxidative stress-induced premature senescence[25]. Previous studies demonstrated that, early-passage FoxM1-/- mouse embryonic fibroblasts (MEFs), immortalized FoxM1+/+ MEFs, and pancreata from mice with a pancreas-specific knockout of FoxM1 displayed premature senescence, indicating the prevention of premature senescence by FoxM1[24-26]. Accordingly, silencing of FoxM1 by RNA interference in U2OS cells resulted in cellular senescence[28]. We have also discovered that in HepG2 and SMMC-7721 HCC cells, knockdown of FoxM1 led to cellular senescence, whereas the senescent cells were decreased by FoxM1 overexpression[11]. Likewise, in this study, to elucidate the functional relevance of FoxM1 in GBC, SA-β-gal staining was performed to determine the senescent cells. Our data suggested that lower expression of FoxM1 in GBC-SD cells was associated with more senescent cells, indicating the involvement of FoxM1 in the prevention of senescence in GBC.

In conclusion, as FoxM1 was highly expressed in GBC, our results for the first time demonstrated that down-regulation of FoxM1 expression in GBC-SC cells by RNA interference could inhibit cell proliferation, invasion and induce cellular senescence, providing clear evidence that FoxM1 is a potential therapeutic target for the treatment of GBC.

COMMENTS

Background

The incidence of gallbladder carcinoma is currently rising faster in Oriental countries than Western world, though the etiology is largely unknown. Progression of this disease is associated with a proliferation-associated transcription factor Forkhead box M1 (FoxM1), a crucial molecular in cell cycle progression.

Research frontiers

FoxM1 overexpression is common in most malignant tumors of the digestive system. However, whether FoxM1 is also up-regulated in gallbladder carcinoma, and the specific roles of FoxM1 in gallbladder carcinoma have not been unequivocally addressed. In this study, the authors demonstrate that overexpression of FoxM1 could be a potential mechanism for mediating cellular senescence and viability in gallbladder carcinoma.

Innovations and breakthroughs

Recent reports have highlighted the importance of proliferation-associated transcription factors, including FoxM1, in gastrointestinal carcinogenesis. Our previous study reported that FoxM1 was also overexpressed in gallbladder carcinoma, and that FoxM1 expression was closely correlated with GBC differentiation, Nevin stage and metastasis. Furthermore, this in vitro studies suggest that this protein may be the cause of the repression of senescence observed in this malignancy, and that silencing this protein significantly impaired the cellular viability in gallbladder carcinoma, suggesting that it is a potential therapy target.

Applications

By understanding how FoxM1 is functionally involved in cellular viability in gallbladder carcinoma and by blocking its expression, this study proposes a future strategy for therapeutic intervention in the treatment of patients with gallbladder carcinoma.

Terminology

Cellular senescence is an irreversible growth arrest, characterized by decreased cell proliferation, combined with accumulation of mitotic defects and chromosomal instability. This permanent exit from the cell cycle represents an important tumor suppression mechanism. Non-surprisingly, in accordance with its proliferation stimulating function, FoxM1 prevents both oncogene- and oxidative stress-induced premature senescence.

Peer review

The authors examined the functional involvement of FoxM1 in gallbladder carcinoma. This research is well organized and of relevant interest and scientific innovation.

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