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Original Research

Expression of FOXA1 gene regulates the proliferation and invasion of human gastric cancer cells

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Abstract: Forkhead box (FOX) transcription factors regulate the development of several human cancers. However, the role and therapeutic potential of FOXA1 in gastric cancer is still largely unexplored. The results showed a significant ($P < 0.05$) upregulation of FOXA1 in gastric cancer tissues and cell lines. Silencing of FOXA1 in gastric cells significantly ($P < 0.05$) decreased their viability through induction of apoptosis. The induction of apoptosis was associated with upregulation of Bax and downregulation of Bcl-2. Additionally, FOXA1 silencing caused activation of caspase-3 and 9 with no apparent effects on the expression of caspase-8 suggestive of intrinsic apoptosis. The transwell cell invasion revealed significant ($P < 0.05$) decline of cell invasion of gastric cancer cells upon FOXA1 silencing. The FOXA1 knockdown further inhibited the *in vivo* tumor growth suggestive of its therapeutic potential. Taken together, the findings of the present revealed that FOXA1 regulates the proliferation and development of gastric cancer and may exhibit therapeutic implications in gastric cancer treatment.

Key words: Gastric cancer; Forkhead transcription factors; FOXA1; Apoptosis; Transwell assay.

Introduction

The human gastric cancer is the fourth most prevalent type of cancer at the global level (1). In terms of the overall mortality resulting from the cancerous malignancies, gastric cancer is considered as the second most lethal human cancer. As per reports, around 0.7 million deaths result from the gastric cancer worldwide annually (2). During recent years, decline in the incidence rates of gastric cancer has been observed which is believed to be the result of consciousness about nutrition, hygiene and *Helicobacter pylori* infection (2). Also, there has been an advancement in the anti-cancer strategies presently opted against this serious malignancy. At present, the surgical resection in combination with the enhanced procedures of standardized lymphadenectomy is the foremost treatment method used against gastric cancer (3). The major hurdle in the gastric cancer treatment is difficulty of diagnosing the disease at early stages. Therefore, to achieve better clinical outcomes against the gastric cancer and to allow higher survival of the gastric cancer patients, it is necessary to search for the novel prognostic and therapeutic targets. Recent discoveries in molecular oncology have remarkably enhanced our understanding. Identification of diversity of cell signaling pathways has enabled researchers to explore the interconnected regulatory networks which crosstalk to promote the development and tumorigenesis of human cancers (4, 5). The eukaryotic forkhead box (FOX) transcription factors have been shown to regulate vital biological processes such as cell differentiation, cell division, apoptosis, senescence and cell survival (6, 7). Research studies have proved that a number of proteins belonging to FOX gene family play crucial regulatory role in the tumorigenesis of many human cancers. Forkhead box M1 was shown to regulate the invasion and angiogenesis of pancreatic cancer and has been reported to act as the novel target in the cancer therapy (8, 9). Similarly, the forkhead box A1 (FOXA1) transcription factor has been shown to be associated with several human cancers like breast cancer, ovarian cancer and lung cancer (10-12). However, little is known about the regulatory control experienced by FOXA1 in gastric cancer. The present study was designed to examine the expression of FOXA1 in human gastric cancer, to unveil its role and to explore the therapeutic implications of FOXA1 in the management of human gastric cancer.

Materials and Methods

Human tissue specimens

The clinical tissue specimens pertaining to the human gastric cancer and corresponding non-cancer stomach tissues were obtaining from the gastric cancer patients through surgical resection at Department of General Surgery, Liyang Branch, Jiangsu People's Hospital, Liyang, Jiangsu, China. The patients didn't receive any chemotherapeutic treatments prior to the specimen collection. Informed written consents were taken from the patients for experimental usage of the clinical tissues. The research ethics committee of Jiangsu People's Hospital, Liyang, Jiangsu, China approved the study under approval number JPH-645IV-2019 The tissues were transported in liquid nitrogen containers and were stored

at -80°C until their experimental usage. Moreover, the standard ethical procedures were followed strictly for carrying out the experimentation on the specimens.

Culture of cell lines and cell transfection

All the human gastric cancer cell lines (BGC-823, MGC-803, MKN-45 and SGC7901) and the normal gastric epithelial cell line (GES-1) were purchased from the American Type Collection Center (ATCC), USA. All the cell lines were cultured using the RPMI-1640 culture medium (Gibco). The supplementation of fetal bovine serum (FBS, 10%) was used for the cell line culture. The culturing was performed in humidified incubator at 37°C with 5% CO_2. The knockdown construct of FOXA1 gene, si-FOXA1 and the respective silencing control, si-NC were bought from RiboBio Biotech. Co., China. The transfection of cancer cells was done with the help of Lipofectamine 2000 reagent (Thermo Fisher Scientific) as per the manufacturer protocol.

Expression analysis

The extraction of the total RNA from cell lines and tissues was performed with the help of Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's method. Using the reverse transcription kit (Takara Bio Inc.), the synthesis of first strand cDNA was performed. The expression of FOXA1 gene was studied through quantitative real-time PCR (qRT-PCR) method with the help of SYBR Green reagent (Thermo Fisher Scientific). The $2^{-\Delta\Delta Ct}$ cycle threshold method was used for determining the relative expression of FOXA1. Human GADPH gene was used as an internal control in the qRT-PCR expression study.

Cell viability assay

The viability of the MGC-803 and MKN-45 transfected with si-FOXA1 or si-NC was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were seeded in 96-well plates at a density of 1×10^6 cells/well. Around 20 µL of 5 mg/L of the MTT solution was added to each well at 37°C for 4 h. To dissolve the formazan crystals, 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The optical density (OD) was determined at 570 nm for the estimation of cell viability.

Apoptosis assays

The study of apoptosis of gastric cancer cell lines, MGC-803 and MKN-45 transfected with si-FOXA1 or si-NC, was acridine orange/ethidium bromide (AO/EB) dual staining assays. Briefly, the stably transfected cells were plated on 12-well plate at 2.5×10^4 cells per well. The plate was incubated in humidified incubator at 37°C for 48 h. Afterwards, the cells were harvested and washed with phosphate buffered saline (PBS) buffer. The fixing of cells was performed using 70% ethanol and the cells were stained with AO/EB solution. The AO/EB stained cells were visualized with the help of fluorescence microscope. Green, yellow and red cells depict normal, early and late apoptotic cells respectively.

***In vivo* tumorigenesis study in mice**

The in vivo study was performed in 7-8 weeks old female SCID/NOD rat models which were kept in well ventilated and airy rooms in the institute's animal house. The approval for the animal experimentation was taken from the institute's animal ethics committee. The xenografted mice models were developed as described previously (13). Briefly, the mice were subcutaneously injected with MGC-803 (1×10^5) to induce the tumor development. The mice were administered with intra-tumor injections (five in total, each after 3 days) carrying si-NC or si-FOXA1 constructs. After the administration of intra-tumor injections, the mice were sacrificed, and the tumors were excised. The average tumor size (cm), weight (g) and volume (mm^3) were calculated.

Western blot analysis

The transfected cancer cells were lysed on the ice with the help of RIPA lysis and extraction buffer (Thermo Fisher Scientific). The cellular fractions were run on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The PAGE gel was blotted to nitrocellulose membrane. The membrane was given the exposure of anti-Bax, anti-Bcl-2 and anti-actin primary antibodies for 12 h at 4°C. The membrane was again exposed to secondary antibodies conjugated to horseradish peroxidase and finally the detection of protein bands was made using ECS through chemiluminescence.

Immuno-histochemical staining

The immuno-histochemical staining method was used for the analysis of protein levels of Ki67 and cleaved caspase-3. The 4 µm thick tissue sections form mice tumors were firstly stained with hematoxylin and eosin. Afterwards, the immuno-staining of the sections was performed with Ki67 and caspase-3 (cleaved) fluorescent antibodies. The sections were dehydrated using ethanol, mounted on glass slide and visualized using fluorescent microscope.

Statistical analysis

The experiments were performed with three replicas. Data were presented as mean ± standard deviation (SD). Statistical analysis was carried out with the GraphPad Prism 7.0 software. Student's t-test was performed to compare the inter-value differences. $P < 0.05$ was taken to represent the statistically significant difference.

Results

Significant up-regulation of FOXA1 gene in gastric cancer

To understand the expression pattern of FOXA1 gene, qRT-PCR study was performed. It was noticed that the gastric cancer tissues showed significantly ($P < 0.05$) higher FOXA1 expression than the normal gastric tissues (Figure 1A). Further, all the gastric cancer cell lines (BGC-823, MGC-803, MKN-45 and SGC7901) were seen to have markedly higher transcript levels of FOXA1 in comparison to the normal gastric epithelial cell line, GES1 (Figure 1B). The results thus suggest that over-expression of FOXA1 might be acting as one of the pathological cues for the onset and development of human gastric cancer.

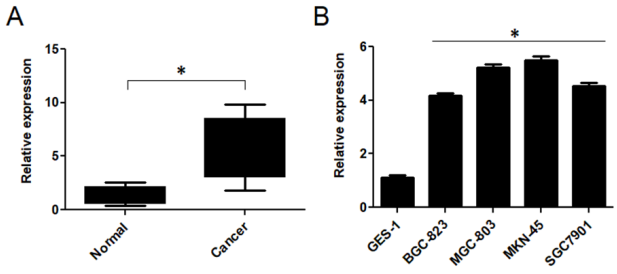

Figure 1. FOXA1 is significantly upregulated in gastric cancer. Quantitative RT-PCR study for expression analysis of FOXA1 gene from (A) non-cancerous and cancerous gastric tissues (B) normal human gastric epithelial cell line (GES-1) and gastric cancer cell lines (BGC-823, MGC-803, MKN-45 and SGC7901). The RT-PCR carried three replicates per reaction and expressed as mean ± SD (*P < 0.05).

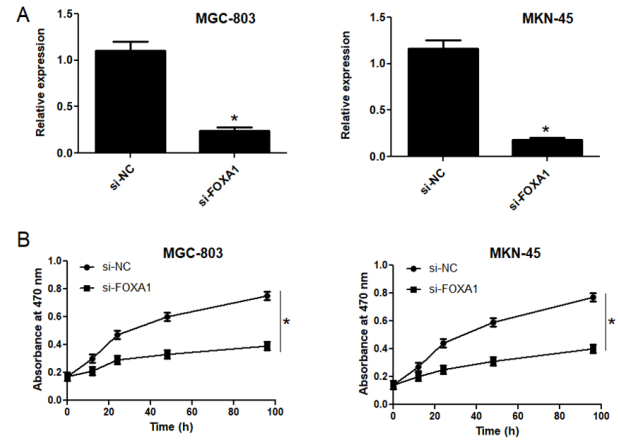

Figure 2. Silencing of FOXA1 inhibits the proliferation of gastric cancer cells (A) Quantitative RT-PCR study for expression analysis of FOXA1 gene from MGC-803 and MKN-45 cancer cells stably transfected with si-NC or si-FOXA1 (B) MTT cell proliferation assay for the analysis of viability of MGC-803 and MKN-45 cancer cells stably transfected with si-NC or si-FOXA1. Three experimental replicates were used for carrying out the experiments and final values were given as mean ± SD (*P < 0.05).

Silencing of FOXA1 inhibits the proliferation of gastric cancer cells

To understand the regulatory role of FOXA1 transcription factor in controlling the vital aspects of gastric cancer, FOXA1 gene expression was silenced in cancer cell lines, MGC-803 and MKN-45. The transcriptional knockdown was confirmed by RT-PCR and both the cell lines were seen to possess significantly lowered gene expression of FOXA1 gene (Figure 2A). Now, in order to investigate whether FOXA1 gene silencing had any effect on the proliferation of the gastric cancer cells, the proliferation of MKN-45 and MGC-803 cell lines under transcriptional repression of FOXA1 was examined using MTT assay. Interestingly, the results showed that the cancer cells proliferated at significantly lower rates under FOXA1 gene down-regulation (Figure 2B). Therefore, the results revealed that up-regulation of FOXA1 in gastric cancer might be responsible for allowing cell proliferation at elevated rates.

Silencing of FOXA1 induces intrinsic apoptosis in gastric cancer cells

Whether the reduction in proliferation of gastric cancer cells under the transcriptional knockdown FOXA1 gene was resulting from the cancer cell apoptosis, the study of apoptosis of MGC-803 and MKN-45 cancer cells was undertaken under FOXA1 down-regulation. Similar inferences were drawn from acridine orange/ethidium bromide (AO/EB) staining. The cancer cells showed visual nuclear lesions under FOXA1 gene silencing (Figure 3A). The western blotting of Bax and Bcl-2 apoptosis marker proteins showed that both MGC-803 and MKN-45 cancer cell lines possessed higher Bax protein level under the silencing of FOXA1 gene while they exhibited considerably lower Bcl-2 protein expression. Moreover, FOXA1 silencing induced the cleavage of caspase-3 and 9 with no apparent effects on the expression of caspase-8 suggestive of intrinsic apoptosis (Figure 3B). The results thus reveal that the induction of cancer cell apoptosis due to transcriptional repression of FOXA1 gene inhibited the cell growth showing the therapeutic utility of this crucial regulator in gastric cancer.

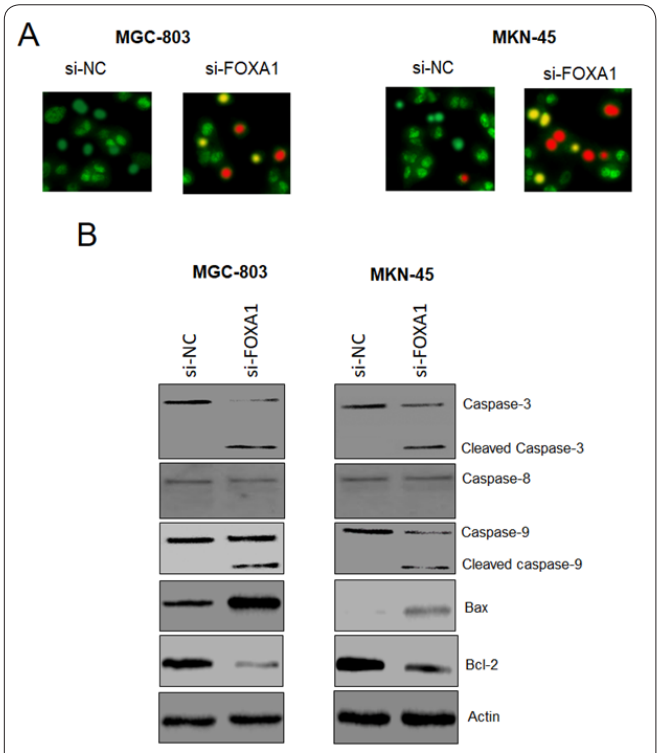

Figure 3. Silencing of FOXA1 induces intrinsic apoptosis in gastric cancer cells. (A) Analysis of apoptosis of MGC-803 and MKN-45 cancer cells stably transfected with si-NC or si-FOXA1 using AO/EB staining procedure. Green, yellow and red cells depict normal, early and late apoptotic cells respectively (C) expression analysis of Caspse-3, 8, 9, BAX and Bcl-2 apoptosis marker proteins from MGC-803 and MKN-45 cancer cells stably transfected with si-NC or si-FOXA1. Three independent replicates were used for conducting the experimental procedures.

Silencing of FOXA1 inhibits the invasion of gastric cancer cells

One of the important aspects of the cancer pathogenesis is the invasiveness of the proliferating cells to invade and infect the surrounding tissues. To enquire if FOXA1 has any regulatory role in controlling this critical process, the *in vitro* invasion analysis through transwell assay was carried out. It was found that the cancer cell lines, MGC-803 and MKN-45 exhibited significantly lower invasion rate under FOXA1 knockdown in

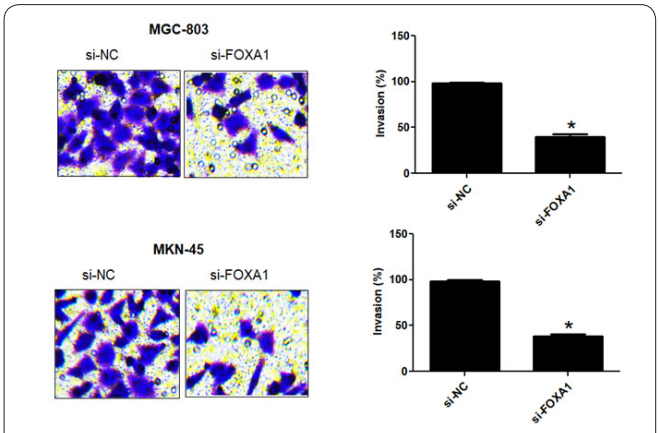

Figure 4. Silencing of FOXA1 inhibits the invasion of the gastric cancer cells. Assessment of *in vitro* invasion of MGC-803 and MKN-45 cancer cells stably transfected with si-NC or si-FOXA1. The experiments were performed using three independent replicates and percent cell invasion was determined from manual cell counting from 7 random microscopic fields (*P < 0.05).

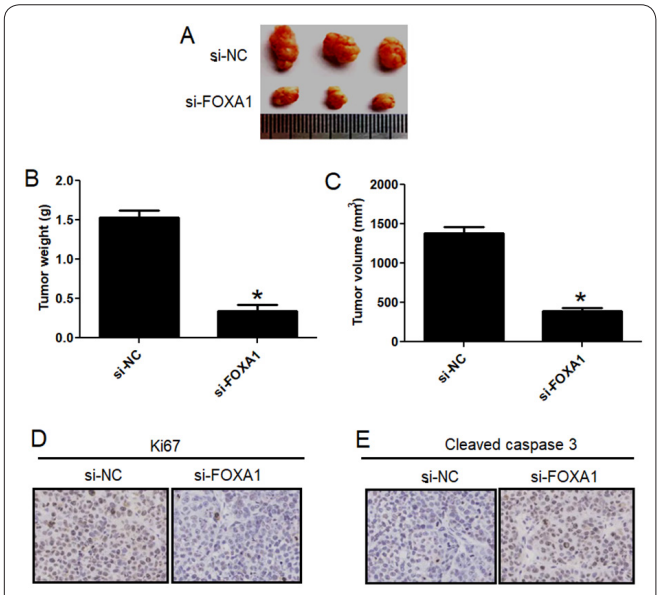

Figure 5. The *in vivo* tumor growth was significantly reduced under FOXA gene silencing. (A) Morphological comparison of mice tumors from rat models receiving si-NC or si-FOXA1 intra-tumor injections (B) mice tumor weight (g) under administration of si-NC or si-FOXA1 intra-tumor injections (C) mice tumor volume (mm^3) under the administration of rat models by si-NC or si-FOXA1 intra-tumor injections (D) immuno-histochemical staining analysis of Ki67 proliferation marker from tumor sections under the administration of si-NC or si-FOXA1 intra-tumor injections (E) immuno-histochemical staining analysis of cleaved caspase-3 protein from tumor sections under the administration of si-NC or si-FOXA1 intra-tumor injections. Each experimental method was carrying three replicas and difference was considered statistically significant only at $*P < 0.05$.

comparison to the normal control cancer cells (Figure 4). The gastric cancer cell invasion was found to be reduced by more than 50% under the down-regulation of FOXA1. Therefore, FOXA1 might be utilized as crucial therapeutic target against the gastric cancer progression.

Silencing of FOXA1 inhibits the tumor growth *in vivo*

Lastly to see whether the *in vitro* findings about the regulatory control experienced by FOXA1 in gastric cancer correlates with its effects *in vivo,* the cancer cells were injected into the mice models. After the tumor onset the mice were administered with intra-tumor injections carrying either the knockdown construct of FOXA1 (si-FOXA1) or its negative control (si-NC). The mice tumors were excised after sacrificing the animals and their morphological assessment was made where it was found that mice tumors were significantly lower in size in comparison under FOXA1 silencing (Figure 5A). Moreover, the average tumor weight (in grams) was also found to be significantly lesser for mice treated with si-FOXA1 intra-tumor injections (Figure 5B). Similarly, the volume of the mice tumors was also markedly lower under the transcriptional knockdown of FOXA1 gene (Figure 5C). Interestingly, the tumor sections when processed for immuno-histochemical fluorescence staining, the expression of proliferation marker Ki67 was found to be fairly lower in tumor sections obtained for the mice tumors in which FOXA1 silencing was performed (Figure 5D). On the other hand, the levels of cleaved caspase-3 protein were significantly higher in such mice tumors revealing higher cellular apoptosis (Figure 5E). Taken together, the *in vivo* study results clearly suggest that FOXA1 plays a crucial regulatory role in the molecular mechanics of gastric cancer and suggest the possibility of utilizing the therapeutic potential of FOXA1 as an alternative anti-cancer approach against human gastric cancer.

Discussion

Human gastric cancer is one of the lethal human malignancies and is responsible for a significant number of cancer related deaths, worldwide. The current treatment strategies are less effective, and thus the cancer management becomes very difficult as the patients diagnosed with this disease exhibit extensive tumor progression and metastasis of the lymphatic tissue (14, 15). As such, it is needed to explore the prognostic and therapeutic measures against the human gastric cancer. In an attempt towards this, we in the present study showed that forkhead box A1 (FOXA1) transcription factor is significantly over-expressed in gastric cancer tissues and cells. The dysregulation of FOXA1 was previously reported to be linked with other human cancers also (16, 117). The silencing of the FOXA1 gene in the gastric cancer cells led to the inhibition of the cell growth through induction of intrinsic apoptosis. The induction of apoptosis was also observed in liver cancer cells when FOXA1 was silenced, and our results also reflect the similar role of FOXA1 in human cancer (18). There are several reports that FOXA1 gene controls the metastasis of the human cancer cells. The non-small lung cancer cells exhibited lower migration and invasion potential under the transcriptional knockdown of FOXA1 gene (19). To ascertain whether FOXA1 exerts similar regulatory control on the motility of the gastric cancer cells, the transcriptional knockdown of FOXA1 was performed. It was found that the results matched with the previous findings and the repression of FOXA1 in gastric cancer cells markedly reduced their *in vitro* invasion potential. The therapeutic value of FOXA1 in gastric cancer was disclosed in depth by its ability to re-

gulate the tumor size in rat models *in vivo*. The silencing of FOXA1 led to significant decline in the tumor growth and progression in mice. The tumor sections were seen to exhibit lower cell division rate as the proliferation marker Ki67 expression was significantly lower. The Ki67 is considered as an important biomarker of proliferation and lower expression of Ki67 in si-FOXA1 transfected cells is suggestive of the growth inhibitory effects exerted by FOXA1 silencing (20). On the other hand, the cleaved caspase-3 is an important marker for the induction of apoptosis (21) and the levels of cleaved caspase-3 protein were significantly higher in such mice tumors indicative of higher cellular apoptosis. Taken together, the results of the present work are suggestive of the key regulatory role of forkhead box A1 (FOXA1) in maintaining the growth and progression of gastric cancer and explored the possibility of utilizing FOXA1 gene in gastric cancer prognosis and treatment.

Collectively, the study revealed that gastric cancer exhibits significant upregulation of FOXA1 gene expression. The experimental knockdown of FOXA1 resulted in the inhibition of the growth of gastric cancer via induction of intrinsic apoptotic both *in vitro* and *in vivo*. Moreover, knockdown of FOXA1 declined the cell invasion gastric cancer cells suggestive of the therapeutic value of FOXA1 in the management of human gastric cancer.

Competing interests

The authors declare no competing interests.

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