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

Yun Dai¹, Guangming Yang¹, Lie Yang¹, Li Jiang¹, Guohua Zheng¹, Shuyin Pan¹, Chuming Zhu*²

¹Department of Pathology, Liyang Branch, Jiangsu People's Hospital, Liyang, Jiangsu, 213300, China
²Department of General Surgery, Liyang Branch, Jiangsu People's Hospital, Liyang, Jiangsu, 213300, China

*Correspondence to: chumingzhu6@yahoo.com
Received July 14, 2021; Accepted August 11, 2021; Published August 31, 2021
Doi: http://dx.doi.org/10.14715/cmb/2021.67.2.25
Copyright: © 2021 by the C.M.B. Association. All rights reserved.

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 Bel-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 study 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-6451V-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% CO₂. 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^{-ΔΔCt} 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 1×10⁴ 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 fixing of cells was performed using 70% ethanol, mounted on glass slide and visualized using fluorescent microscope.

**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 2.5×10⁴ 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 × 10⁶) 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 (mm³) 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 horse-radish 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.
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 can-
cer 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.

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
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 a 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-
FOXA1 regulates gastric cancer development.

Yun Dai et al.

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.

References

1. Sitarz R, Skierucha M, Mielko J, Offerhaus GJ, Maciejewski R, Polkowski WP. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res 2018; 10:239.
2. Eusebi LH, Telese A, Marasco G, Bazzoli F, Zagari RM. Gastric cancer prevention strategies: A global perspective. J Gastroenterol Hepatol 2020; 35: 1495-1502
3. Vergari R, Polenta V, Marmolare C. Cancer of the Stomach, In Management of Elderly Patients, Springer, Cham 2018, pp. 179-190.
4. Farooqi AA, Naureen H, Attar R. Regulation of cell signaling pathways by circular RNAs and microRNAs in different cancers: Spotlight on Wnt/β-catenin, JAK/STAT, TGF/SMAD, SHH/GLI, NOTCH and Hippo pathways. Semin Cell Dev Biol 2021; S1084-1710 (21) 00075-00076.
5. Farooqi AA, Nayyab S, Martinelli C, Berardi R, Katifelis H, Gazouli M, Cho WC. Regulation of Hippo, TGFβ/SMAD, Wnt/β-Catenin, JAK/STAT, and NOTCH by Long Non-Coding RNAs in Pancreatic Cancer. Front Oncol 2021; 11:657965
6. Lam EW, Brosens JJ, Gomes AR, Koo CY. Forkhead box proteins: tuning forks for transcriptional harmony. Nat. rev Cancer 2013; 13(7): 482-495.
7. Kaestner KH, Knöchel W, Martinez DE. Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev 2000; 14(2): 142-146.
8. Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH. Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer Res 2007; 67(17): 8293-300.
9. Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH. Forkhead box M1 transcription factor: a novel target for cancer therapy. Cancer Treat Rev 2010; 36(2): 151-156.
10. Wang K, Guan C, Fang C, Jin X, Yu J, Zhang Y, Zheng L. Clinical significance and prognostic value of Forkhead box A1 expression in human epithelial ovarian cancer. Oncol Lett 2018; 15(4): 4457-4462.
11. Hirata K, Takakura Y, Shibazaki M, Morii M, Honda T, Oshima M, Aoyama K, Iwama A, Nakayama Y, Takano H, Yamaguchi N. Forkhead box protein A1 confers resistance to transforming growth factor-β-induced apoptosis in breast cancer cells through inhibition of Smad3 nuclear translocation. J Cell Biochem 2019; 120(2): 2259-2270.
12. Huang C, Liu J, Xiong B, Yonemura Y, Yang X. Expression and prognosis analyses of forkhead box A (FOXA1) family in human lung cancer. Gene 2019; 685: 202-210.
13. Tang Y, Geng Y, Luo J, Shen W, Zhu W, Meng C, Li M, Zhou X, Zhang S, Cao J. Downregulation of ubiquitin inhibits the proliferation and radioresistance of non-small cell lung cancer cells in vitro and in vivo. Sci Rep 2015; 5(1): 1-2.
14. Dassen AE, Lemmens VE, Van De Poll-fransv LE, Cremers GM, Brenninkmeijer SJ, Lips DJ, Vd Wurf AA, Bosscha K, Coebergh JW. Trends in incidence, treatment and survival of gastric adenocarcinoma between 1990 and 2007: a population-based study in the Netherlands. Eur J Cancer.2010; 46(6): 1101-110.
15. Wu HH, Lin WC, Tsai KW. Advances in molecular biomarkers for gastric cancer: miRNAs as emerging novel cancer markers. Expert Rev Mol Med 2014; 16: e1.
16. Guu J, Moller C, Charon-Barra C, Boissiere F, Crapez E, Chartron E, Lamy PJ, Gutowski M, Bourgier C, Romieu G, Simony Lafontaine J. Prognostic value of androgen receptor and FOXA1 co-expression in non-metastatic triple negative breast cancer and correlation with other biomarkers. Br J Cancer 2018; 119(1): 76-79.
17. Schrijver W, Schuurman K, van Rossum A, Droog M, Jeronimo C, Salta S, Henrique R, Wesseling J, Moelans C, Linn SC, van den Heuvel M. FOXA1 levels are decreased in pleural breast cancer metastases after adjuvant endocrine therapy, and this is associated with poor outcome. Mol Oncol 2018; 12(11): 1884-1894.
18. Gan HY, Lin N, Zhang Q, Feng ZZ. Silencing FOXA1 gene regulates liver cancer cell apoptosis and cell proliferation. Oncol Lett 2018; 15(4): 4457-4462.
19. Li J, Zhang S, Zhu L, Ma S. Role of transcription factor FOXA1 in non small cell lung cancer. Molecular medicine reports. 2018 Jan 1;22(2):397-404.
20. Li J, Zhang S, Zhu L, Ma S. Role of transcription factor FOXA1 in non small cell lung cancer. Molecular medicine reports. 2018 Jan 1;22(2):397-404.
21. Bressenot A, Marchal S, Bezdetnaya L, Garrier J, Guiellemin F, Plénet F. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. J Histochem Cytochem 2009; 57(4): 289-300.