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Forkhead box A1 expression in thyroid carcinoma based on bioinformatics and clinical significance

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Thyroid cancer, known to be common in women than men, accounts for 12% all types of cancers and ranks 9th with 5,86,202 cases worldwide. Role of forkhead box A1 (FOXA1), also known as hepatocyte nuclear factor 3α and involved in the oncogenesis and progression of several tumors such as gliomas, breast, stomach, lung, ovarian, and esophageal cancers, has not been elucidated well in thyroid carcinoma until now. Here, we analyzed the expression of FOXA1 in thyroid carcinoma tissues and its effects on the biological characteristics of papillary thyroid carcinoma TPC-1 cells. The expression levels of FOXA1 in normal thyroid and thyroid carcinoma tissues were analyzed using UALCAN database (http://ualcan.path.uab.edu/index.html), and the correlations between FOXA1 expression levels and survival time of patients with thyroid carcinoma were analyzed. Si-FOXA1 and si-NC were transfected into cells that were then divided into si-FOXA1 (transfected si-FOXA1), si-NC (transfected si-NC), and control (TPC-1) groups. Cell proliferation was determined with MTT assay, and invasion ability was measured by Transwell assay. Early apoptotic rate was detected by flow cytometry. The mRNA expression levels of p27Kip1, Cyclin D1 and Cyclin E were measured by qRT-PCR. The expression level of FOXA1 was significantly higher in thyroid carcinoma tissues than that in normal thyroid tissues. UALCAN-based analysis indicated that patients with low expression level of FOXA1 had longer survival time than those with high expression level of FOXA1 (P <0.05). The cell proliferation rate was significantly lower in si-FOXA1 group than those in si-NC group and control group at 24 h, 48 h and 72 h (P <0.05). The early apoptotic rate was significantly higher and number of invading cells was lower in si-FOXA1 group than those in si-NC and control groups (P <0.05). Si-FOXA1 group had higher mRNA expression level of p27Kip1 and lower expression levels of Cyclin D1 and Cyclin E than those of si-NC and control groups (P <0.05). Targeted inhibition of FOXA1 suppresses the proliferation and invasion and promotes the apoptosis of thyroid carcinoma cells, probably by regulating activation of the p27Kip1 pathway.

Keywords: Hepatocyte nuclear factor, Thyroid gland, Tumor

Thyroid carcinoma (TC) is a common solid malignancy derived from the endocrine system. Accounting for 12% of all types of cancers in adolescents and 2% in children globally, it ranks 9th with 586 202 new thyroid cancer cases worldwide in 2020. However, thyroid cancer shows highest survival rate, up to >99%. The US has recorded 43,800 fresh cases in 2020 with women reporting three times higher compared to men[1-3]. Thyroid cancer incidence in China is nearly double the world average incidence registering 11.3 per 100 000 persons[4]. Although thyroid cancer ranks the seventh most common cancer in China, its mortality is still quite low[5]. In Shanghai, thyroid cancer incidence has increased rapidly in recent years, jumping to the second most common cancer in women[6]. The projection of thyroid cancer burden from time to time makes critical information available to plan effective measures for its prevention and management.

Forkhead box A (FOXA) family involves liver-specific transcription factors, including FOXA1, FOXA2 and FOXA3. Among them, FOXA1 participates in epithelial-to-mesenchymal transition as well as liver, lung, prostate and pancreas development, which is of great significance for the formation of mammary ducts[7,8]. Studies have demonstrated that FOXA1 is involved in regulating the occurrence of lung carcinoma, brain tumors, endometrial carcinoma, breast carcinoma and prostate carcinoma[9,10]. FOXA1 is considered as an essential factor in the process of nuclear hormone receptor signal transduction. In this study, we searched the Cancer Genome Atlas for FOXA1 expression in papillary thyroid carcinoma tissues and adjacent normal tissues, and tried to analyze

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its correlation with the prognosis of TC patients. Meanwhile, FOXA1 expression was downregulated using cell transfection technology, and its effects on the biological characteristics of TC cells were explored, so as to investigate the role of FOXA1 in the occurrence and progression of papillary thyroid carcinoma and its significance for survival and prognosis.

Materials and Methods

General data

The expression of FOXA1 in normal thyroid tissues and TC tissues was analyzed using UALCAN database (http://ualcan.path.uab.edu/index.html), and its correlation with survival time of TC patients was also investigated.

Experimental cells, main reagents and apparatus

TPC-1 cells were provided by the Shanghai Cell Bank of Chinese Academy of Sciences. Small interference (si)-FOXA1 and si-negative control (NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. (China). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FOXA1 antibody (Beyotime Institute of Biotechnology, Shanghai, China) and Western blotting kit (Beijing Aobox Biotechnology Co., Ltd., China) were used. Transwell chamber (Nanjing Jiancheng Bioengineering Institute, China), CO_2 cell incubator (Forma Scientific, USA), ultra-clean workbench (Jiangsu Haimen Kylin Medical Instrument Factory, China), microscope (Olympus, Japan), vertical gel electrophoresis system and gel imaging analysis system (Bio-Rad Laboratories, USA), and magnetic stirrer (Ningbo Scientz Biotechnology Co., Ltd., China) were employed.

Cell transfection and grouping

Frozen TPC-1 cells were resuscitated, inoculated in RPMI 1640 medium, and incubated in an incubator at 37°C with 5% CO_2. Later, cells in the logarithmic growth phase were selected, transfected with si-FOXA1 (si-FOXA1 group) and si-NC (si-NC group) according to the instructions of the Lipofectamine™ 2000 Transfection Reagent, and then cultured routinely. In addition, a control group was set, and the cells were treated with routine culture without transfection.

Detection of FOXA1 expression level in cells by Western blotting

TPC-1 cells in the logarithmic growth phase were inoculated into a 6-well plate at a density of 5×10^5 cells/well. The cell grouping was the same as that in 1.3, with 8 replicate wells in each group. The cells were incubated in an incubator at 37°C with 5% CO_2. After the cells were lysed in accordance with the kit instructions, they were subjected to electrophoresis to separate the protein, followed by color development with diaminobenzidine and gel imaging. Image processing software (ImageJ) was adopted for statistical analysis. GAPDH was used as a reference for the expression of the target protein.

Determination of cell viability by MTT assay

TPC-1 cells in the logarithmic growth phase were inoculated into a 96-well plate at a density of 1×10^5 cells/well. The cell grouping was the same as mentioned above, with 8 replicate wells in each group. After the cells were incubated in an incubator at 37°C with 5% CO_2 for 24 h, 20 μL of MTT solution was added to each well, followed by incubation at room temperature. The absorbance (A) at 450 nm was detected using a multifunctional microplate reader (Multiskan MK3) at 6, 12, 18 and 24 h after incubation. Cell viability = $(A_{\text{wells in experimental group}} - A_{\text{wells in control group}})/ A_{\text{wells in experimental group}} \times 100\%$.

Determination of cell apoptosis rate by flow cytometry

TPC-1 cells in the logarithmic growth phase were inoculated into a 6-well plate at a density of 1×10^6 cells/well. The cell grouping was the same as mentioned above, with 8 replicate wells in each group. The cells were incubated in an incubator at 37°C with 5% CO_2. After incubation for 24 h, the cells were re-suspended in 200 μL of binding buffer. After 10 μL of Annexin V-FITC and PI were added, the cells were incubated at room temperature for 15 min in the dark. Then, cell apoptosis was detected by flow cytometry. Late apoptotic and dead cells as well as early apoptotic cells were located in the upper right area and lower right area on the flow cytometry diagram, respectively.

Determination of cell invasion ability by Transwell assay

TPC-1 cells in the logarithmic growth phase were inoculated in the serum-free medium in the upper chamber of the Transwell chamber (a layer of Matrigel was spread on the Transwell plate) at a density of 3×10^5 cells/well. The cell grouping was the same as that in 1.3, with 8 replicate wells in each group. Meanwhile, the medium containing 10% fetal bovine serum was added to the lower chamber, and the cells were incubated in an incubator at 37°C with 5% CO_2. After incubation for 24 h, the cells on the top surface of the membrane were wiped off, and

stained with crystal violet at room temperature. Subsequently, the cells on the bottom surface of the membrane were observed under a microscope, and 5 visual fields were randomly selected for cell count, the average of which was taken.

Detection of mRNA expression by RT-qPCR

Total RNA in the corresponding tissues and cells was extracted using TRIzol reagent, and reversely transcribed into cDNA using the reverse transcription kit, followed by PCR amplification, with U6 gene as an internal reference. Smart View gel imaging was used to take photos for analysis and detect the expression of long noncoding (lnc) RNA CASC2, or detect the expressions of lnc RNA CASC2 and miR-634 using the SYBR PCR Master Mix kit. The reaction conditions of qRT-PCR involved: pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 50 s, annealing at 60°C for 25 s, and extension at 72°C for 15 s. The expression levels of p27Kip1, Cyclin D1 and Cyclin E were calculated by $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: p27Kip1 forward primer: 5'-CTTTACCCGGATGGACCTCG-3', reverse primer: 5'-CTTGCCAATGACCCTCCATA-3', Cyclin E forward primer: 5'-GCCATCTGAAGGCAA-ACGC-3', Cyclin D1 forward primer: 5'-CAAG-CCCTACAATTTTTA CATCG-3', reverse primer: 5'-CCTGCTTCACCTCC TTCTCATA-3', Cyclin E forward primer: 5'-GCCAT CTGAAGGCAAACGC-3', reverse primer: 5'-GCCA ATGGTTGTCTCAC-AGAAC-3', and U6 forward primer: 5'-CTCG-CTTCGGCAGCACA-3', reverse primer: 5'-AAC-GCTTCACGAATTTGCGT-3'.

Statistical analysis

SPSS 26.0 software was adopted for statistical analysis, and GraphPad Prism 5.01 was software used for plotting. Measurement data related to cell proliferation, migration and invasion conforming to normal distribution were expressed as mean ± standard deviation ($\bar{\chi} \pm s$), and independent *t*-test was employed for comparison between two groups. P <0.05 indicated that a difference was statistically significant.

Results

Prediction of FOXA1 expression in TC by bioinformatics

The expression of FOXA1 was significantly higher in TC tissues than that in normal thyroid tissues. Moreover, UALCAN-based analysis indicated that patients with a low expression of FOXA1 had a higher survival time curve than those with a high expression of FOXA1, showing a statistically

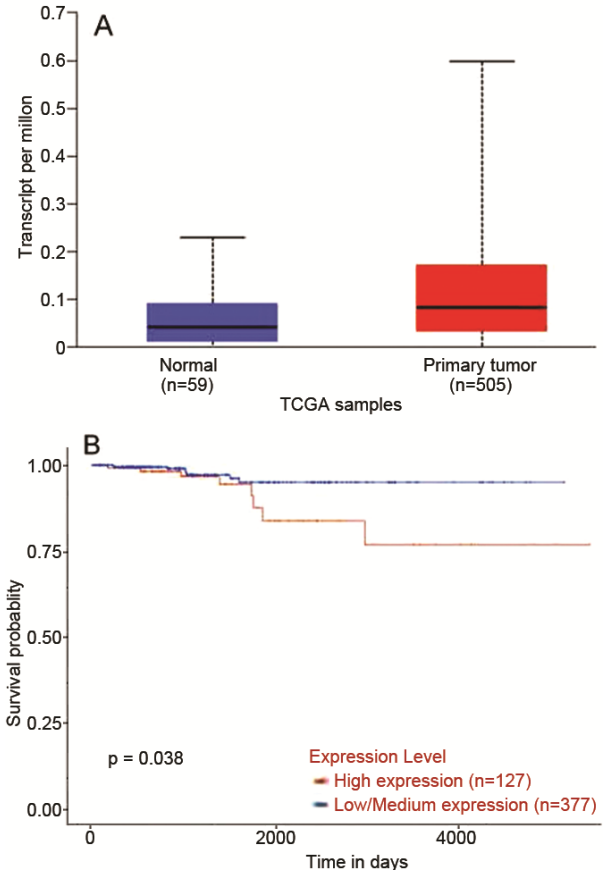

Fig. 1 — Prediction of FOXA1 expression in TC by bio-informatics. (A) Analysis of FOXA1 expression in normal thyroid tissues and TC tissues; and (B) Analysis of correlation between FOXA1 expression and survival time of TC patients using UALCAN database.

significant difference (P <0.05) (Fig. 1). The results confirmed that FOXA1 may serve as an oncogene in TC.

Construction of TPC-1 cells stably over-expressing FOXA1

The expression of FOXA1 was significantly lower in si-FOXA1 group than that in si-NC group and control group (P <0.001) (Fig. 2), indicating that a cell line that silences FOXA1 has been successfully constructed.

Effect of downregulating FOXA1 on proliferation of TPC-1 cells

The cell proliferation rate was significantly lower in si-FOXA1 group than that in si-NC group and control group at 24, 48 and 72 h (P <0.05) (Fig. 3), suggesting that downregulating FOXA1 notably inhibits cell proliferation.

Effect of downregulating FOXA1 on apoptosis and of TPC-1 cells

The early apoptotic rate of cells was significantly higher in si-FOXA1 group than that in si-NC group

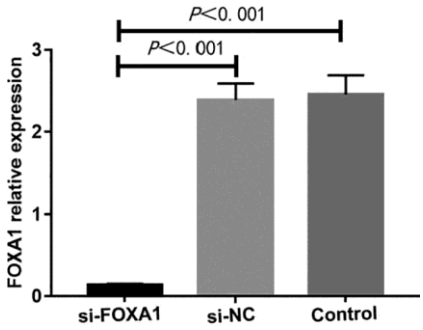

Fig. 2 — Detection of FOXA1 expression in cells by Western blotting

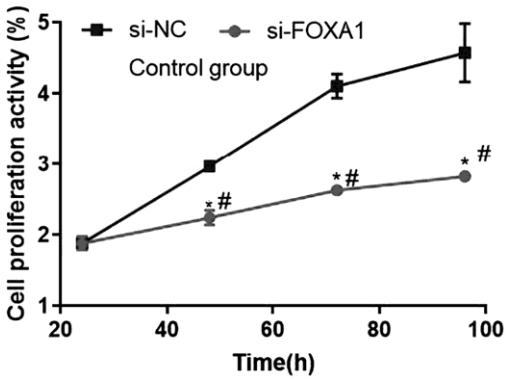

Fig. 3 — Effect of downregulation of FOXA1 on proliferation of TPC-1 cells. [*P <0.05 *vs.* si-NC group, #P <0.05 *vs.* control group]

and control group (P <0.05) (Fig. 4), demonstrating that downregulating FOXA1 remarkably promotes cell apoptosis.

Effect of downregulating FOXA1 on invasion of TPC-1 cells

The number of invasive cells was significantly lower in si-FOXA1 group than that in si-FOXA1 group and control group (P <0.05) (Fig. 5), displaying that downregulating FOXA1 markedly inhibits cell invasion.

Effect of downregulating FOXA1 on mRNA expression levels of p27Kip1, Cyclin D1 and Cyclin E in TPC-1 cells

Si-FOXA1 group had a higher relative mRNA expression level of p27Kip1 and lower relative mRNA expression levels of Cyclin D1 and Cyclin E than si-NC group and control group (P <0.05) (Fig. 6).

Discussion

In recent years, TC has become the most common malignancy occurring in the head and neck regions, but it is superior to other types of malignant tumors in terms of prognosis, which is attributed to the comprehensive tumor classification that provides references for clinical treatment. Furthermore, specific tumor markers are conducive to the early diagnosis of TC[11,12].

The FOX family has 17 sub-families with more than 100 members, which are extensively involved in various biological processes such as embryo formation and cell growth, differentiation, development, metabolism, immunity, and cycle regulation[13,14]. Recent studies have confirmed that the FOX family, especially FOXA1, is closely related to

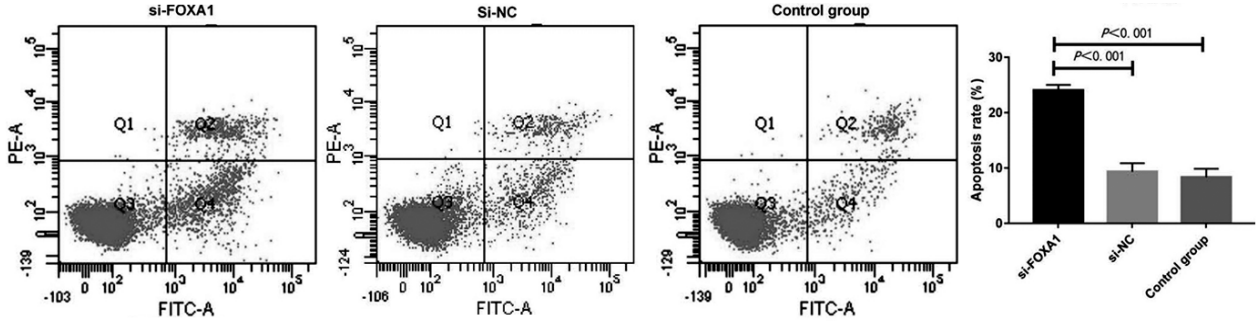

Fig. 4 — Effect of downregulation of FOXA1 on apoptosis of TPC-1 cells.

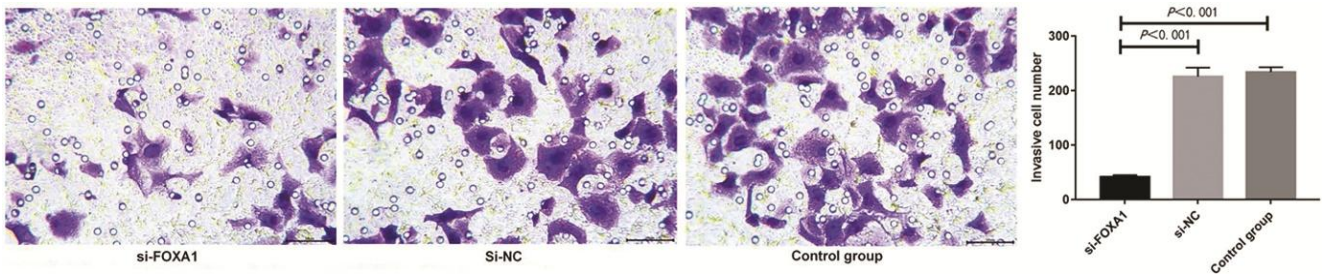

Fig. 5 — Effect of downregulation of FOXA1 on invasion of TPC-1 cells

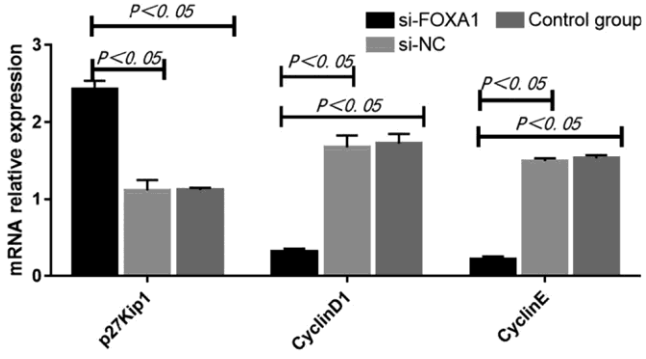

Fig. 6 — Effect of downregulation of FOXA1 on mRNA expression levels of p27Kip1, Cyclin D1 and Cyclin E in TPC-1 cells.

the occurrence and progression of tumors. The FOXA1 gene is located on human chromosome 14q21.1, and its structure includes an N-terminal transcription activation domain, a DNA-binding FOX domain in the middle, and a C-terminal activation transcription binding domain related to histone H3/H4[15,16]. FOXA1 can be demethylated by replacing histone H1 and dimethylation of H3 lysine 4 and DNA genomes, thus loosening the dense chromatin region bound to it and promoting transcription, so it is called the pioneer factor[17,18]. FOXA1 is expressed in breast, esophagus, liver, pancreas, bladder, prostate, colon and lung tissues, which participates in metabolic processes, signal regulation, and the initiation of cell cycle-related genes, and is closely associated with the pathogenesis of multiple tumors such as lung carcinoma, esophageal carcinoma, prostate carcinoma and breast carcinoma [19,20]. In this study, the relevant data of the bioinformatics database were analyzed, and the results showed that the expression of FOXA1 was significantly higher in TC tissues than that in normal thyroid tissues. Moreover, UALCAN-based analysis indicated that patients with a low expression of FOXA1 had a higher survival time curve than those with a high expression of FOXA1, confirming that FOXA1 may serve as an oncogene in TC. SiRNA can target and inhibit FOXA1 expression in TC cells. Cancer cell malignant proliferation is an important biological behavior in the occurrence and progression of malignant tumors[21], and inhibiting cell proliferation is the key to the targeted treatment of malignant tumors. In order to explore the effects of FOXA1 expression on the biological characteristics of TC, TPC-1 cell line was selected as the research object in this study. Therefore, FOXA1 expression was regulated using cell transfection technology, and the results indicated that down-regulating FOXA1 can significantly inhibit the proliferation and invasion and promote the apoptosis of TPC-1 cells.

p27Kip1 is a tumor suppressor gene that is closely related to a variety of malignancies, and its biological activity is to hinder the expressions of cyclins such as Cyclin D1 and Cyclin E, thus suspending the cell cycle, and exerting tumor inhibition effect. A previous study showed that FOXA1 can inhibit the tumor-inhibition activity of p27Kip1, and accelerate cell cycle and promote cell proliferation after inhibiting p27Kip1[22,23]. In this study, the changes in the gene expression of the p27Kip1 pathway in TC cells after targeted inhibition of FOXA1 were analyzed. Si-FOXA1 group had a higher relative mRNA expression level of p27Kip1 and lower relative mRNA expression levels of Cyclin D1 and Cyclin E than si-NC group and control group (P <0.05), indicating that targeted inhibition of FOXA1 can promote the activation of the p27Kip1 signaling pathway in TC cells. Considering the proliferation-promoting activities of downstream Cyclin D1, Cyclin E and other cell cycle molecules of the p27Kip1 signaling pathway, it was speculated that promoting the activation of the p27Kip1 signaling pathway might be a molecular mechanism for inhibiting the proliferation and invasion of TC cells after targeted inhibition of FOXA1.

Conclusion

The above study has demonstrated that the expression level of FOXA1 is significantly higher in thyroid carcinoma tissues than that of the normal thyroid tissues. UALCAN-based analysis indicated that patients with low expression level of FOXA1 had longer survival time than those with high expression level of FOXA1 (P <0.05). Targeted inhibition of FOXA1 can suppress the proliferation and invasion and promote the apoptosis of TC cells by regulating the activation of the p27Kip1 pathway.

Conflict of interest

Authors declare no competing interests.

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