

Six1 Overexpression Promotes Glucose Metabolism and Invasion Through Regulation of GLUT3, MMP2 and Snail in Thyroid Cancer Cells

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Introduction: Sineoculis homeobox homolog 1 (Six1) overexpression has been implicated in several human cancers. To date, its clinical significance and potential function in human thyroid cancer remain unclear.

Methods: Immunohistochemistry was used to examine the protein expression of BCAT1 in 89 cases of thyroid cancer tissues. We overexpressed and knockdown Six1 in TPC-1 and B-CPAP thyroid cancer cell lines. Biological roles and potential mechanisms of Six1 were examined using CCK-8, colony formation assay, Matrigel invasion assay, Western blot, PCR, ATP assay, and 2-NBDG uptake assay.

Results: We showed that Six1 protein was upregulated in thyroid cancers and was associated with tumor size and nodal metastasis. Analysis of TCGA dataset indicated that Six1 mRNA was higher in thyroid cancers compared with normal thyroid. CCK-8, colony formation and Matrigel invasion assays demonstrated that Six1 overexpression promoted proliferation, colony number and invasion while Six1 siRNA knockdown inhibited the growth rate, colony formation ability and invasive ability in both cell lines. Notably, Six1 upregulated glucose consumption, lactate production level and ATP level. 2-NBDG uptake analysis showed that Six1 overexpression upregulated glucose uptake while Six1 knockdown inhibited glucose uptake. Further analysis revealed that Six1 overexpression upregulated Snail, MMP2 and GLUT3 at both mRNA and protein levels. TCGA analysis demonstrated positive associations between Six1 and Snail, MMP2 and GLUT3 at the mRNA levels.

Conclusion: Taken together, our data demonstrated that Six1 was upregulated in human thyroid cancers and promoted cell proliferation and invasion. Our data also revealed new roles of Six1 in thyroid cancer development by modulating glucose metabolism and invasion, possibly through regulation of Snail, MMP2 and GLUT3.

Keywords: Six1, thyroid cancer, glucose metabolism, GLUT3

Introduction

Thyroid cancer is a common endocrine cancer world wide and its incidence has increased in the past decade.¹ The major subtypes of non-medullary thyroid cancer includes papillary thyroid cancer, follicular thyroid cancer, anaplastic thyroid cancer and poorly differentiated thyroid cancer. Papillary thyroid cancer accounts for more than 80% of all thyroid cancer cases and the development of distant metastases is a major contributing factors to restricted prognosis.² It is important to understand the mechanism of thyroid carcinogenesis, proliferation, invasion and metastasis, which may provide insights into potential treatment strategies.³

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Sineoculis homeobox homolog 1 (Six1) is a homeodomain-containing transcription factor. Six1 is highly expressed during embryo development and plays important roles in mammalian organ development.⁴⁻⁶ Six1 enhances progenitor cell survival and its absence leads to the reduction in size or loss of many organs and increased apoptosis.⁷⁻¹⁰ The expression of Six1 remains at a low level in adult tissue and its overexpression contributes to tumor initiation.¹¹ It has been found that Six1 was overexpressed in several human malignancies such as breast,^{12,13} cervical,¹⁴ ovarian,¹⁵ and pancreatic cancers. Six1 overexpression enhances cancer proliferation, survival and epithelial-mesenchymal transition (EMT).^{16,17} However, its clinical significance and biological functions in human thyroid cancer remain elusive.

In the present study, we examined its expression pattern in thyroid cancer tissues. We then overexpressed and depleted Six1 in thyroid cancer cell lines. The biological function and underlying mechanism of Six1 in thyroid cancer was also investigated.

Patients and Methods

Patients and Specimens

The study protocol was approved by the Institutional Reviewer Board of Shengjing Hospital. Thyroid cancer tissues were obtained from 89 thyroid cancer patients who received surgical operation between 2013 and 2016 in the Shengjing Hospital. Normal thyroid tissues were from benign thyroid disease such as hyperplasia. Written informed consent was provided by the patients. The study was conducted in accordance with the Declaration of Helsinki. The histological diagnosis were evaluated according to the World Health Organization (WHO) classification guidelines.¹⁸

Immunohistochemical Staining

4 μ m thick sections of formalin-fixed, paraffin-embedded tumor samples were obtained from Pathology Archive. Immunohistochemical staining was carried out using the Elivision Plus staining kit from MaiXin (MaiXin, Fuzhou, China). Briefly, sections were deparaffinized in xylene, and rehydrated in alcohol and water. Antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) for 2 minutes in an autoclave. Hydrogen peroxide (0.3%) was used to block peroxidase. Then sections were then incubated with Six1 antibody (1:200 dilution rate, Sigma HPA00189, USA) at 4°C for 10 hours. After washing with

PBS, section was incubated with HRP polymer from Elivision Plus staining kit at room temperature. Staining was developed using DAB.

We selected five views (containing >80% tumor cells) per section randomly to analyze Six1 intensity. Nuclear Six1 staining score was scored as 0 (negative), 1 (moderate), 2 (strong). The percentage score was divided as 1: 1–25%, 2: 26–50% 3: 51–75% and 4: 76–100%. Intensity score and percentage scores were multiplied to obtain a Six1 staining score of 0 to 8. Six1 expression was considered as low when the staining score <4; Six1 expression was considered as high (overexpression) when the staining score \geq 4.

Cell Culture and Transfection

Thyroid cancer cell lines B-CPAP and TPC-1 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). pCMV-SIX1 transfection plasmid was obtained from Origene. siRNA sequences for Six1 and scramble siRNA were purchased from Dharmacon. Lipofectamine 3000 (Invitrogen) was used for plasmid transfection and Dharmafect 1 (Dharmacon) was used for siRNA transfection.

Western Blot

For Western blot, 60 μ g protein was separated by SDS-PAGE. Then proteins were transferred to PVDF membrane and incubated with primary antibody including Six1 (1:800; Sigma, USA), MMP2, GLUT3, Snail (1:1000; Cell Signaling Technology, USA), and GAPDH (1:2000; Santa Cruz, USA). Membranes were washed with TBS-T, and incubated with HRP-conjugated secondary antibody (1:2000, Santa Cruz, USA) for 1 hour. Images were taken using the DNR Imaging Systems after ECL development (ThermoFisher, USA).

Realtime PCR

RNA was isolated using RNAiso (TAKARA). For quantitative PCR cDNA was synthesized using the iScriptTM Reverse Transcription Supermix (Bio-Rad). Realtime PCR was performed by ABI 7500 PCR system using SYBR Green Master Mix (TAKARA). The thermal profile is 50°C for 2min, 95°C for 2 min, 45 cycles of 95°C for 15 sec and 60°C for 40 sec. β -actin was used as the endogenous calibrator. Relative fold change was calculated using 2- $\Delta\Delta$ Ct method. Experiments were done in triplicated. The primer sequences are as follows: Six1 forward, 5'

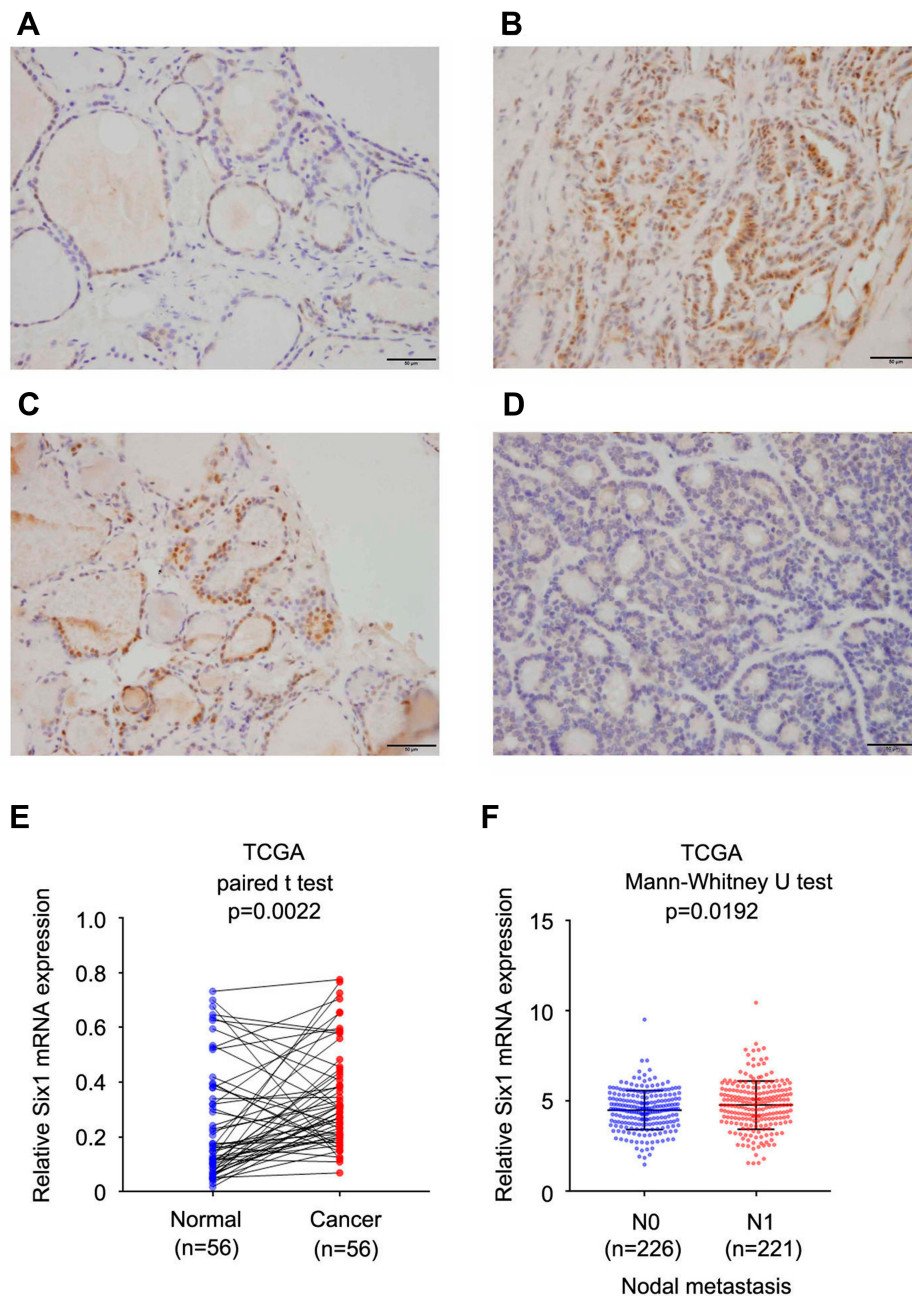


Figure 1 Expression Six1 in thyroid cancer tissues. (A) Negative/weak staining of Six1 in normal thyroid tissues. (B) Positive nuclear Six1 expression in a case of papillary thyroid cancer. (C) Positive nuclear Six1 staining in a case of follicular thyroid cancer. (D) Negative staining a case of follicular thyroid cancer. (E) TCGA data showed that Six1 mRNA was elevated in thyroid cancers compared with normal tissue (paired t test, $p=0.0022$). (F) TCGA data showed Six1 mRNA was higher in cancers with positive nodal metastasis (Mann-Whitney U-test, $p=0.0192$). Bar indicates 50 μ M.

AAGGAGAAGTCGAGGGGTGT 3', Six1 reverse, 5' TGCTTGTGGAGGAGGAGTT 3'; MMP2 forward, 5' TGTGTTCTTTGCAGGGAATGAAT 3', MMP2 reverse, 5' TGTCTTCTGTTTTGCTCCAGTT 3'. GLUT3 forward, 5' CCTTTGGCACTCTCAACCAGC 3', GLUT3 reverse, 5' AACCCAGTAGCAGCGGCCAT 3'. Snail forward, 5' GCCCACCTCCAGACCCAC 3', Snail reverse, 5' GCAGGGACATTCGGGAGAA 3'. β -actin forward, 5'

ATAGCACAGCCTGGATAGCAACGTAC 3', β -actin reverse, 5' CACCTTCTACAATGAGCTGCGTGTG 3'.

CCK-8 and Colony Formation Assays

CCK-8 assay: Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded into 96-well plates at about 3000 cells per well. 10 μ L CCK-8 reagent was added into each well.

After incubation at 37°C for additional 2 h, cell proliferation was determined by examining the absorbance at a wavelength of 450 nm using a microplate reader (Bio-Rad, USA). Colony formation was performed using 3000 cells per culture dish. Then the cells were cultured for about 15 days then stained using Giemsa. Experiments were done in triplicated.

Matrigel Invasion Assay

Matrigel invasion assay used a 24 well Transwell chamber (Corning, 8 µm pore size) with 20 µL of Matrigel (BD Bioscience) was added to the chamber at a 1:3 dilution rate. Cells were transferred to the upper chamber in serum-free medium. 10% FBS 1640 medium was added to the lower chamber. After 18 hours cells remaining on the upper chamber surface were removed. The experiments were performed in triplicate.

Glucose Uptake

Glucose uptake was examined with using 2-NBDG. Cells were incubated with 1mM 2-NBDG in 1640 medium at 37°C for 30 minute. Cells were washed with PBS and analyzed on flow cytometer at 488 nm excitation. The experiments were performed in triplicate.

Analysis of ATP Production, Glucose Consumption, and Lactate Level

Glucose and lactate assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ATP level was examined with the Beyotime ATP Assay Kit (Guangzhou, China). After transfection, the supernatants were harvested, and glucose and lactate levels in the medium were determined using glucose and lactate assay kits according to the manufacturer's instructions. The experiments were performed in triplicate.

Statistical Analysis

Statistical analysis was performed using SPSS 17. Six1 mRNA (RNA-seq data) of thyroid cancer was obtained from The Cancer Genome Atlas (TCGA). The Mann-Whitney *U*-test was used to compare Six1 mRNA in cancers with/without nodal metastasis. Paired *t*-tests were used to compare Six1 mRNA in cancers and normal thyroid tissue. A χ^2 test was used to examine possible correlations between Six1 expression and clinicopathologic factors. Student's *t*-test was used to compare data obtained

from biological experiments. A $p < 0.05$ was considered as statistically significant.

Results

Six1 Is Upregulated in Human Thyroid Cancers

A total of 89 thyroid cancer cases (including 71 papillary carcinoma, 9 follicular carcinoma and 9 anaplastic carcinoma) and 20 normal thyroid tissues were used for analysis of Six1 protein expression. Negative or weak nuclear Six1 staining was observed in normal thyroid tissues (Figure 1A) while upregulated Six1 nuclear staining was found in 46 out of 89 cases of thyroid cancer specimens (Figure 1B–D). The high Six1 level was positively associated with tumor size ($p = 0.0027$) and nodal status ($p = 0.0330$) (Table 1). There was no significant association between Six1 with age ($p = 0.1561$), sex ($p = 0.713$) and histological subtype ($p = 0.5057$). A summary of immunohistochemical results was provided in [Supplementary Table 1](#).

We also analyzed of the TCGA data from thyroid cancers. As shown in Figure 1E&F, expression of Six1 mRNA was elevated in thyroid cancers compared with normal tissue (paired *t* test, $p < 0.05$, Figure 1E). Six1 mRNA levels were higher in cancers with positive nodal metastasis

Table 1 Association of SIX1 Expression with Clinicopathological Parameters in Patients with Thyroid Cancer

Characteristics	Number of Patients	SIX1 Low Expression	SIX1 High Expression	P
Age (years)				
<45	49	27	22	0.1561
≥45	40	16	24	
Sex				
Male	18	8	10	0.7130
Female	71	35	36	
Tumor size (cm)				
<2	54	33	21	0.0027
≥2	35	10	25	
Lymph node metastasis				
Absent	54	31	23	0.033
Present	35	12	23	
Tumor types				
Papillary	71	33	38	0.5057
Follicular	9	6	3	
Anaplastic	9	4	5	

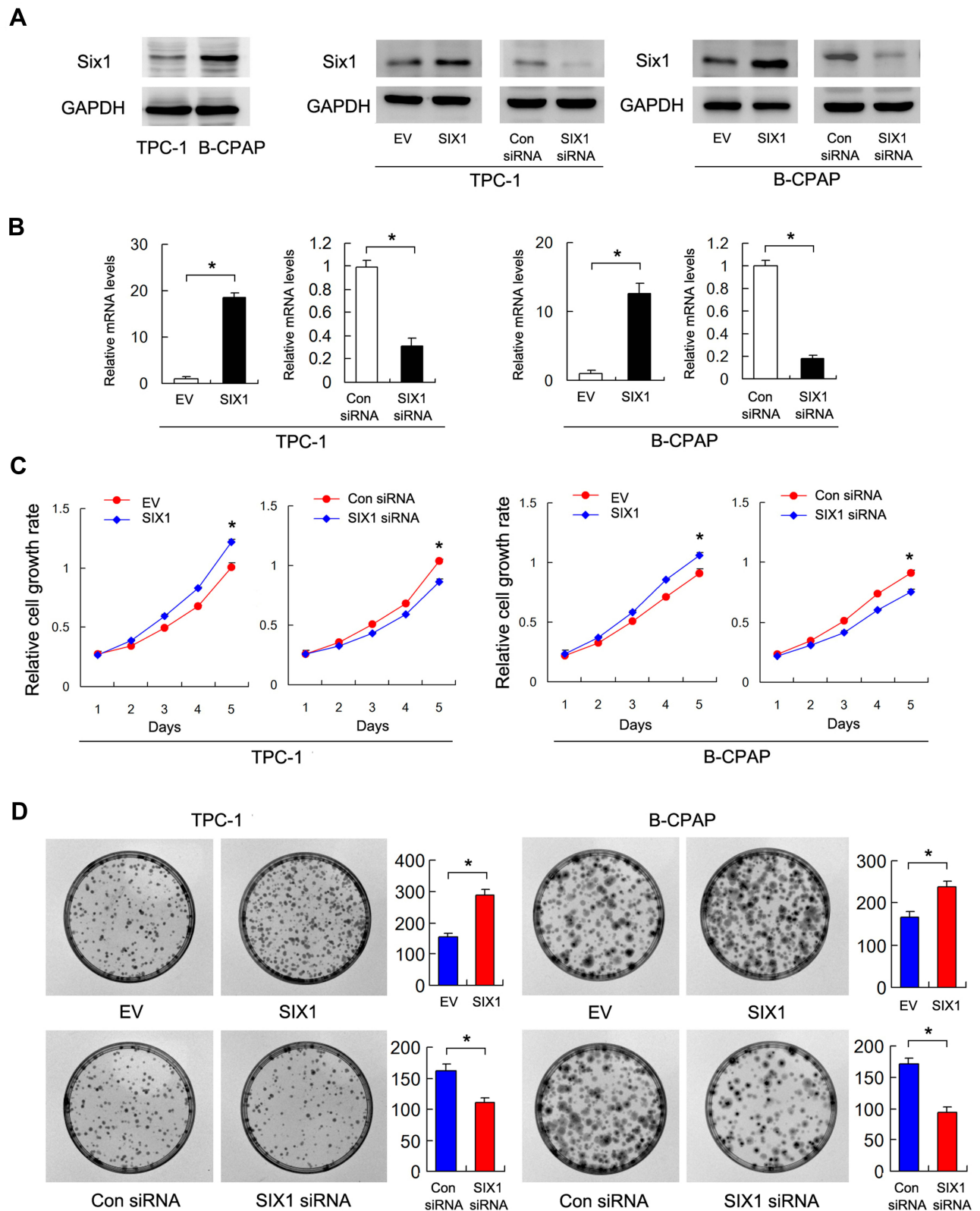


Figure 2 Six1 regulates cell proliferation in thyroid cancer cells. **(A)** Six1 protein expression in a 3 cell lines. Western blot showed efficiencies of Six1 plasmid transfection and siRNA knockdown in TPC-1 and B-CPAP cell lines. **(B)** Realtime PCR showed that Six1 transfection and siRNA knockdown efficiency in both TPC-1 and B-CPAP cell lines. **(C)** CCK-8 assay showed that that Six1 depletion downregulated the proliferation rate while Six1 overexpression upregulated proliferation rate in both TPC-1 and B-CPAP cells. **(D)** Colony formation assay demonstrated that Six1 overexpression upregulated colony number while Six1 depletion downregulated colony number in both cell lines. * $p < 0.05$.

(Mann–Whitney *U*-test, $p < 0.05$, Figure 1F). Taken together, these data indicated that Six1 was upregulated in human thyroid cancers and correlated with malignant features.

Six1 Promotes Proliferation and Invasion

Six1 protein expression was examined in 2 thyroid cancer cell lines (TPC-1, B-CPAP). We transfected both B-CPAP and TPC-1 cells with the Six1 plasmid and siRNA. Transfection efficiency was confirmed by RT-qPCR and Western blots (Figure 2A and B). The CCK-8 assay demonstrated that Six1 depletion downregulated the proliferation rate of both TPC-1 and B-CPAP cells while Six1 overexpression upregulated proliferation rate (Figure 2C). Colony formation results showed that Six1 transfection increased colony numbers while siRNA treatment decreased colony numbers (Figure 2D). To evaluate the effect of Six1 on invasion, Matrigel invasion assay was performed and the results showed that Six1 increased invading

ability while its depletion decreased invading ability of thyroid cancer cells (Figure 3A).

Six1 Regulates Glucose Uptake and ATP Levels

Glucose metabolism is important for ATP production, cell survival and proliferation. To investigate whether Six1 could modulate glucose metabolism in thyroid cancer cell lines, we examined several steps involved in glucose metabolism including glucose uptake, consumption and lactate production levels.

We used 2-NBDG to evaluate the rate of glucose uptake. As shown in Figure 3B, Six1 overexpression increased the rate of glucose uptake in both TPC-1 and B-CPAP cell lines, while Six1 depletion downregulated the rate of glucose uptake in both cell lines. We also examined the levels of glucose and

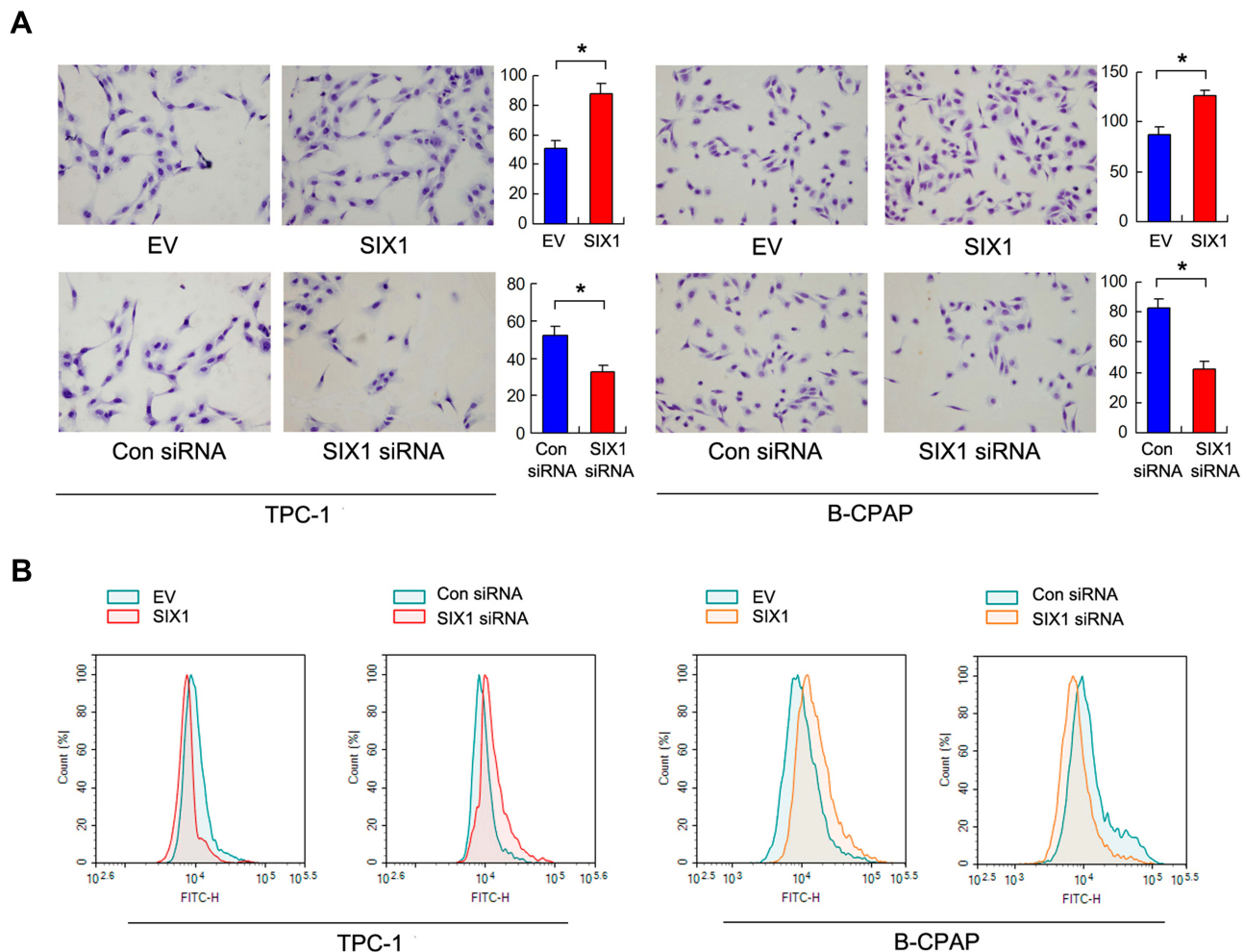


Figure 3 Six1 regulates invasion and glucose uptake. **(A)** Matrigel invasion assay demonstrated that Six1 overexpression increased invading cell numbers, while Eya2 knockdown decreased invading cell numbers in both TPC-1 and B-CPAP cell lines. **(B)** Glucose take assay using 2-NBDG staining and flow cytometry demonstrated that Six1 overexpression enhanced glucose uptake in while siRNA knockdown inhibited glucose uptake in both TPC-1 and B-CPAP cell lines. * $p < 0.05$.

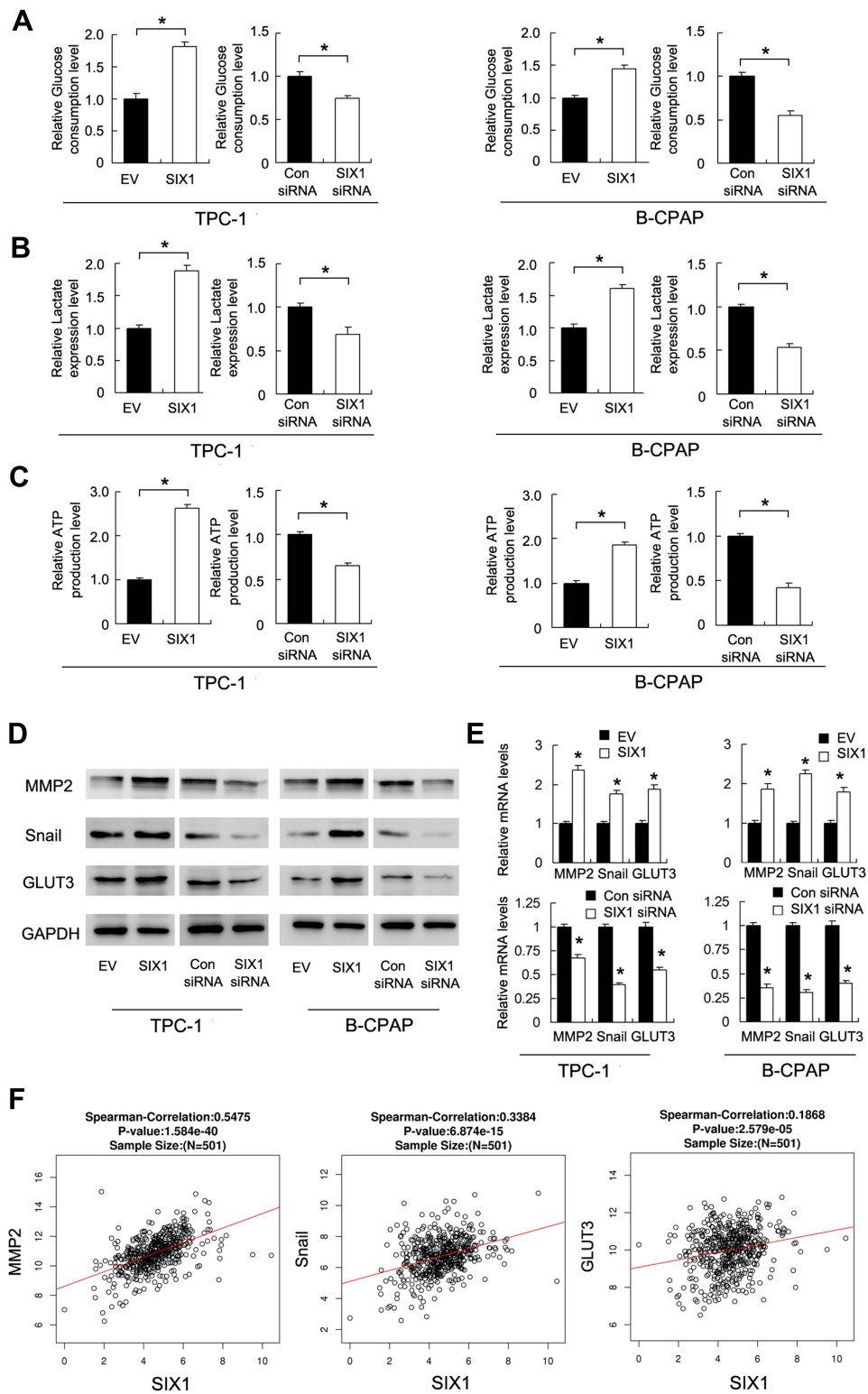


Figure 4 Six1 regulates glucose metabolism and GLUT3, Snail, MMP2 expression. **(A)** Relative glucose consumption rate in thyroid cancer cells transfected with Six1 plasmid and siRNA. **(B)** Relative lactate production rate in thyroid cancer cells transfected with Six1 plasmid and siRNA. **(C)** Relative ATP production level in cells with Six1 plasmid and siRNA. **(D)** Protein levels of MMP2, Snail and GLUT3 in thyroid cancer cells transfected with the Six1 plasmid and siRNA. **(E)** The mRNA levels of MMP2, Snail and GLUT3 in thyroid cancer cells transfected with Six1 plasmid and siRNA. **(F)** Pearson's correlation analysis showing positive associations between Six1 and MMP2, Snail and GLUT3 mRNA in 501 cases of thyroid cancer tissues (TCGA dataset). *p<0.05.

lactate in the medium. As shown in Figure 4A and B, Six1 overexpression increased the level of glucose consumption and lactate production while Six1 depletion exhibited the opposite effects, indicating that Six1 was a positive regulator of glucose metabolism in thyroid cancer cells.

Finally, we examined the role of Six1 on intracellular ATP levels. As shown in Figure 4C. Six1 overexpression increased the level of ATP while Six1 depletion down-regulated the level of intracellular ATP in both cell lines, suggesting Six1 enhanced thyroid cancer development through regulation of glucose uptake.

Six1 Regulates GLUT3, Snail and MMP2 Expression

To identify the mechanism of Six1 on glucose metabolism and invasion, we screened potential related genes. Western blot and realtime PCR demonstrated that Six1 positively regulated GLUT3, Snail, and MMP2 protein and mRNA expression in both TPC-1 and B-CPAP cell lines (Figure 4D and E). Next the association of Six1 with GLUT3, Snail and MMP2 mRNA in thyroid cancer tissues was investigated through analysis of RNA-seq data from TCGA dataset. There was a statistically significant association between Six1 mRNA and GLUT3, Snail, MMP2 mRNA expression (Pearson's correlation, Figure 4F). These data supported our results that Six1 positively regulated glucose metabolism and invasion.

Discussion

Recently, several reports showed that Six1 is upregulated in various human cancers such breast cancer, ovarian cancer, colorectal and hepatocellular carcinoma.^{15,16,19-22} However, its expression pattern and biological roles in human thyroid cancers remain unexplored. Our data demonstrated that Six1 protein expression was increased in thyroid cancer tissues and was positively correlated with tumor size and nodal status. In addition, our results were supported by TCGA data, suggesting Six1 as a potential thyroid cancer biomarker.

Matrigel invasion results showed that Six1 overexpression promoted thyroid cancer invasion, which was in accord with the TCGA data showing positive correlations between Six1 mRNA and nodal metastasis. We further demonstrated that Six1 could upregulate MMP2 and Snail at mRNA and protein levels. MMP2 is required to mediate cancer cell invasion. Snail is an important regulator of epithelial-to-mesenchymal transition (EMT) which can inhibit cell

adhesion by downregulating E-cadherin.^{23,24} MMP2 and Snail were also reported to promotes thyroid cancer progression through induction of cell invasion. TCGA analysis revealed a positive association between Six1 and MMP2/Snail mRNA in thyroid cancer tissues. These data suggested that Six1 functions as a promoter of thyroid cancer invasion possibly through Snail/MMP2 regulation.

The CCK-8 and colony formation assays confirmed the growth promoting role of Six1 in thyroid cancer cells. Cancer cells are dependent on glucose metabolism to produce ATP, which is essential for the malignant growth.²⁵ Glucose transport is the initial step in glucose metabolism. We found that SIX1 overexpression increased glucose uptake into cancer cells with and elevated ATP production, indicating SIX1 as a positive regulator of glucose metabolism in thyroid cancers. We also found Six1 was able to increase mRNA and protein expression of GLUT3. The GLUT family plays critical roles during glucose transport and has been found to be overexpressed in various cancers.²⁶⁻²⁸ Upregulation of GLUT family genes has been reported to be correlated with poor prognosis in papillary thyroid cancers.²⁷ Analysis of a TCGA dataset also confirmed the positive correlation between GLUT3 and Six1 mRNA in 501 cases of thyroid cancers, which further support the role of Six1 on glucose metabolism.

In conclusion, our data demonstrated that Six1 is overexpressed in thyroid cancers and is correlated with nodal metastasis and tumor size. We also linked its oncogenic role with glucose metabolism and invasion, possibly through regulation of MMP2, Snail and GLUT3. These findings suggest Six1 as a potential therapeutic target in thyroid cancers.

Disclosure

The authors report no conflicts of interest in this work.

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