FOXP4 promotes laryngeal squamous cell carcinoma progression through directly targeting LEF-1

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Abstract. Forkhead box (FOX) proteins are multifaceted transcription factors that have been shown to be involved in cell cycle progression, proliferation and metastasis. FOXP4, a member of the FOX family, has been implicated in diverse biological processes in tumor initiation and progression. However, the molecular mechanisms of FOXP4 in laryngeal squamous cell carcinoma (LSCC) remain unknown. In the present study, differentially expressed transcripts in transforming growth factor-β-treated TU177 cells were screened using microarrays and it was found that FOXP4 was significantly upregulated. The high expression of FOXP4 was detected in LSCC tissues and cells, and predicted poor prognosis. The role of FOXP4 in laryngeal cancer cell proliferation, migration and invasion was determined by gain- and loss-of-function assays. Besides, FOXP4 was demonstrated to participate in the epithelial-mesenchymal transition process at the mRNA and protein levels. Mechanically, FOXP4 directly bound to the promoter of lymphoid enhancer-binding factor 1 and activated Wnt signaling pathway, which was confirmed via chromatin immunoprecipitation and luciferase reporter assays. Consequently, these findings provided novel mechanisms of FOXP4 in LSCC progression, which may be considered as potential therapeutic and prognostic targets for LSCC.

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Introduction

Laryngeal squamous cell carcinoma (LSCC) represents >90% of histological subtypes of laryngeal carcinoma (1). The incidence and mortality of laryngeal cancer were 177,422 and 94,771 cases worldwide in 2018 (2). Despite major improvements in diagnosis and treatment, nearly 60% of patients present at an advanced (III or IV) stage when diagnosed (3). Unfortunately, the 5-year survival rate of laryngeal cancer has decreased from 66 to 63% over the past 40 years (4). Hence, it is of importance to identify the exact molecular mechanisms and effective therapeutic strategies of LSCC.

Epithelial-mesenchymal transition (EMT) is a fundamental developmental process during tumor progression characterized by the loss of epithelial characteristics and the gain of mesenchymal features, ultimately leading to the acquisition of enhanced migratory and invasive capacities (5). Among the multiple inducers of EMT, transforming growth factor-β (TGF-β) plays a key role in initiating and driving EMT (6). Previous reports have identified that FOXP4 is involved in EMT process and facilitates invasion and metastasis in breast cancer and hepatocellular carcinoma (7,8). However, whether FOXP4 can promote EMT in LSCC remains unclear, and the potential regulatory mechanism of FOXP4 in this process needs to be clarified.

Forkhead box (FOX) proteins are a superfamily of transcriptional regulators that have been demonstrated to be implicated in cancer initiation, maintenance, progression and drug resistance (9). FOXP4, a member of the FOXP subfamily, is located on human chromosome 6p21.1 and encodes 680 amino acids protein (7). It has been demonstrated that FOXP4 is significantly upregulated in breast cancer, hepatocellular carcinoma and oral squamous cell carcinoma, and was observed to participate in tumorigenesis and progression through various molecular mechanisms (7,8,10). However, the expression level and functional role of FOXP4 in LSCC has not yet been characterized.

In the current study, the expression of FOXP4, its potential functional role in TGF-β-induced EMT and the downstream regulatory mechanisms of FOXP4 were explored in the pathogenesis of LSCC.

Materials and methods

Bioinformatics analysis. FOXP4 was predicted using the Search Tool for Gene Expression Profiling Interactive Analysis (GEPIA) online dataset (http://gepia.cancer-pku.cn/index.html). FOXP4 was identified as a transcription factor of LEF-1 using the Animal TFDB 3.0 database (https://ngdc.cncb.ac.cn/databasecommons/database/id/8) and JASPAR database (http://jaspar.genereg.net).

Patients and specimens. Tumor tissues and paired adjacent normal tissues (distance from tumor margin, >2 cm) were acquired from 81 patients undergoing surgical resection at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between September 2008 and December 2013. The patients had not received radiotherapy or chemotherapy prior to surgery. Patients provided signed informed consent prior to the study. Each surgical resection specimen was divided into two parts, one part was fixed in 10% neutral formalin solution for the conventional wax block; the other part was placed in a fresh state at -80˚C to extract genomic RNA and DNA. Ethical approval was acquired by the ethics committee of the Fourth Hospital of Hebei Medical University. Information on clinicopathological features and survival data were available from hospital recordings.

Cell culture. Three laryngeal cancer cell lines (TU177, TU686 and TU212) were purchased from American Type Culture Collection. All cell lines were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), while AMC-HN-8 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) under conventional culture conditions. All cell lines were authenticated via DNA fingerprinting using the short tandem repeat (STR) method from Procell Life Science & Technology Co., Ltd. The cDNA of 10 LSCC-adjacent tissues were randomly mixed in equal proportion as the control group (pools). For RNA sequencing, TU177 cells were starved in serum-free medium overnight before the addition of recombinant TGF-β1 (10 ng/ml, R&D Systems, Inc.), and cells were routinely cultured for 7 days at 37˚C in a humidified atmosphere with 5% CO_2. Total RNA from TU177 cells was used for sequencing on a HiSeq 2000 system (Illumina, Inc.). Cuffdiff (version 2.2.1.2) was used to compare the log ratio of Fragments Per Kilobase of transcription per Million mapped reads in the two conditions (11). The differential expression results were constructed by GraphPad Prism7 software (GraphPad Software, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR) assay. Total RNA was extracted from LSCC tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration was quantified using a NanoDrop™ spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) RNA (1 μg) was reverse transcribed at 65˚C for 10 min and 85˚C for 5 min using the Transcriptor Fist Strand cDNA Synthesis kit (Roche Life Science Co., Ltd.). RT-qPCR was performed using GoTaq® qPCR Master Mix (Promega Corporation) in the StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 10 min; followed by 40 cycles at 95˚C for 15 sec, 54˚C for 30 sec and 72˚C for 30 sec. Relative gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (12) normalized to GAPDH. The gene-specific primers are listed in Table SI.

Cell transfection. The small interfering RNAs (siRNAs) specifically targeting FOXP4 were synthesized by Shanghai GenePharma Co., Ltd. TU177 cells were divided into four groups: i) si1-FOXP4 (cells transfected with 5 μg si1-FOXP4; 5'-TGTAGAACTCATGATTCTGGGTT-3'); ii) si2-FOXP4 (cells transfected with 5 μg si2-FOXP4; 5'-CAGAATCATGAGTTCTACAAGTT-3'); iii) si3-FOXP4 (cells transfected with 5 μg si3-FOXP4; 5'-CCTGGGCCAGTTTATCAAATT-3'); and iv) si-NC (cells transfected with 5 μg si-NC; 5'-TTCTCCGAACGTGTCACGTTT-3'). AMC-HN-8 cells were divided into two groups: pcDNA3.1-NC (cells transfected with 4 μg pcDNA3.1-NC vector) and pcDNA3.1-FOXP4 (cells transfected with 4 μg pcDNA3.1-FOXP4 vector). The pcDNA3.1-FOXP4 was constructed by Sangon Biotech Co., Ltd. TU177 and AMC-HN-8 cells were cultured until 70-80% confluence in 6-well plate and were transfected using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After transfection at 37˚C for 6 h, the medium was replaced with complete medium. Following incubation at 37˚C for 48 h, transfection efficiencies were assessed and cells were used for subsequent experiments.

Cell viability assay and clone formation assay. The MTS assay was used to determine the cell viability by using CellTiter96® AQueous One Solution Cell Proliferation assay kit (Promega Corporation). TU177 and AMC-HN-8 cells were seeded in 96-well plates with $1x10^3$ per well following transfection for 24 h. After 0, 24, 48, 72 and 96 h, 20 μl MTS reagent was added to cultured mediums and then cells were incubated for 2 h. The optical density at 490 nm was measured for each well. For the clone formation assay, following transfection for 24 h, $3x10^3$ TU177 or AMC-HN-8 cells per well were routinely cultured for 1 week. The clones were fixed in 4% paraformaldehyde at room temperature for 20 min and then stained with crystal violet solution at room temperature for 20 min. The total clones (≥50 cells) were calculated manually under a microscope.

Transwell migration and invasion assays. For the cell migration assay, $1x10^5$ TU177 or AMC-HN-8 cells were seeded into the upper chamber of a Transwell insert (Costar; Corning, Inc.) with 200 μl serum-free medium, while medium with 600 μl 10% FBS was added into the lower chamber. The migratory cells were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with crystal violet solution at room temperature for 20 min after incubation at 37˚C for 24 h. For the cell invasion assay, the steps were the same as described above, except that the membrane of the upper chamber was pre-coated with Matrigel® Basement Membrane Matrix (Costar; Corning, Inc.). Following a 24-h incubation at 37˚C,

the invading cells on the bottom surface of the filter were fixed with 4% paraformaldehyde at 4˚C for 30 min and stained with crystal violet solution at room temperature for 20 min. Cell migration and invasion were analyzed in three randomly selected fields using a light microscope (magnification, x20).

Western blot analysis. RIPA buffer containing PMSF (Beijing Solarbio Science & Technology Co., Ltd.) was used to extract total protein from transfected cells. The quantification of protein was determined using the BCA Protein Assay Kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.]. The lysates (40 μg) were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (MilliporeSigma). Then, membranes were blocked with 5% skimmed milk at room temperature for 60 min. Following which, the membranes were incubated overnight at 4˚C with specific primary antibodies against: β-actin (1:10,000; cat. no. AC026; ABclonal Biotech Co., Ltd.), E-cadherin (1:1,000; cat. no. E-AB-31261; Elabscience Biotechnology, Inc.), N-cadherin (1:1,000; cat. no. E-AB-64011; Elabscience Biotechnology, Inc.), Vimentin (1:1,000; cat. no. bs-0756R; BIOSS), Twist (1:1,000; cat. no. bs-2441R; BIOSS). After washing, the membranes were incubated at room temperature for 45 min with the appropriate IgG HRP-conjugated secondary antibody (1:10,000; cat. no. SA00001-9; ProteinTech Group, Inc.) and detected with enhanced chemiluminescence (ECL) reagent [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] and analyzed using ImageJ software (version 1.48; National Institutes of Health).

Chromatin immunoprecipitation (ChIP) assay. LSCC cells ($1x10^6$) were crosslinked by 1% formaldehyde (Sigma-Aldrich; Merck KGaA) by centrifugation at 300 x g for 3 min at 25˚C and washed in pre-cooled PBS for 10 min at 25˚C. The formaldehyde was quenched by the addition of glycine (Beijing Solarbio Science & Technology Co., Ltd.). Then three sets of 20-sec pulses were used to obtain chromatin fragments. EZ-Magna ChIP A/G kit (MilliporeSigma; 60 μl) was used to perform the ChIP assay according to the manufacturer's protocols. Subsequently, 5 μg anti-IgG (1:40; cat. no. sc-2025; Santa Cruz Biotechnology, Inc.) or anti-FOXP4 (cat. no. ab242127; Abcam) antibodies were used to immunoprecipitate chromatin fragments at 4˚C overnight. The IgG antibody was used as control. Then the immunoprecipitated DNA was purified using a ChIP DNA purification kit (cat. no. D0033; Beyotime Institute of Biotechnology). The fold enrichment of the DNAs amplified by ChIP was assessed the promoter region of the LEF-1 gene. The recovered DNA fragments were evaluated using RT-qPCR with the following primers: Forward, 5'-CAG CCACGCAAATAGAGCAAGG-3' and reverse, 5'-TCCTGG TTCCTCGGCCCGAGA -3'.

Luciferase reporter assay. The promoter regions (-2,308~+91; -1,782~+91; -1,186~+91) of lymphoid enhancer-binding factor 1 (LEF-1) were respectively subcloned into pGL3-Basic vector (E1761; Promega Corporation), and the recombinant plasmids were sequenced and confirmed. LSCC cells were cotransfected with 2 μg pGL3-LEF-1-luc, 2 ng *Renilla* and 2 μg si-NC or si-FOXP4 using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h post-transfection, the Dual-Luciferase Reporter Assay System (Promega Corporation) was used to determine luciferase activity. Firefly luciferase activity was normalised to *Renilla* luciferase activity.

Statistical analysis. SPSS 22.0 software package (IBM Corp.) was used to analyze the data in this study and the data are presented as the mean ± standard deviation. Differences between the paired tissue samples from patients were assessed using a paired Student's t-test, whereas comparisons between two unpaired groups were determined using an unpaired Student's t-test. Differences between multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Pearson's correlation analysis was used to detect the correlation between FOXP4 and LEF-1 expression. Chi-square test was used to analyze the association between FOXP4 expression level and clinicopathological features. The correlation between patient survival and FOXP4 expression was determined by the Kaplan-Meier method and the difference was analyzed using log-rank tests. Cox regression analysis was performed to determine the independent factors affecting survival. A two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

FOXP4 expression is significantly upregulated and closely related with the survival of patients with LSCC. By scanning the GEPIA online dataset (13), it was found that FOXP4 was expressed at relatively high levels in head and neck squamous cell carcinoma (Fig. 1A). A significantly increased expression level of FOXP4 was observed in all laryngeal cancer cell lines (Fig. 1B). Similarly, FOXP4 expression in 81 pairs of LSCC tissues was significantly higher than that in adjacent normal tissues (Fig. 1C). It was identified that the high FOXP4 expression, defined as 200% higher FOXP4 expression in LSCC tissues, was significantly related to lymph node metastasis, TNM stage and pathological differentiation (Table I). In addition, patients with LSCC with high FOXP4 expression level demonstrated poorer survival rate (Fig. 1D). As analyzed by univariate analysis, lymph node metastasis, TNM stage, pathological differentiation and FOXP4 expression were significantly associated with overall survival (Table II). Furthermore, FOXP4 was an independent prognostic factor for patients with LSCC, as determined via multivariate Cox regression analysis (Table II).

FOXP4 promotes laryngeal cancer cell viability, migration and invasion in vitro. To better understand whether FOXP4 plays a biological role in LSCC development, FOXP4 expression was silenced in TU177 cells with two independent si-FOXP4 sequences, si-FOXP4-1 and si-FOXP4-2 successfully knocked down FOXP4 expression compared with the si-NC group (Fig. 2A). Whereas, AMC-HN-8 cells were transfected with pcDNA3.1-FOXP4 to increase FOXP4 expression, which was successful, as shown in Fig. 2B.

The MTS assay demonstrated that transfection of si-FOXP4 or pcDNA3.1-FOXP4 led to a significant inhibition or promotion of laryngeal cancer cell viability, respectively (Fig. 2C). The effects of FOXP4-knockdown were only apparent up to

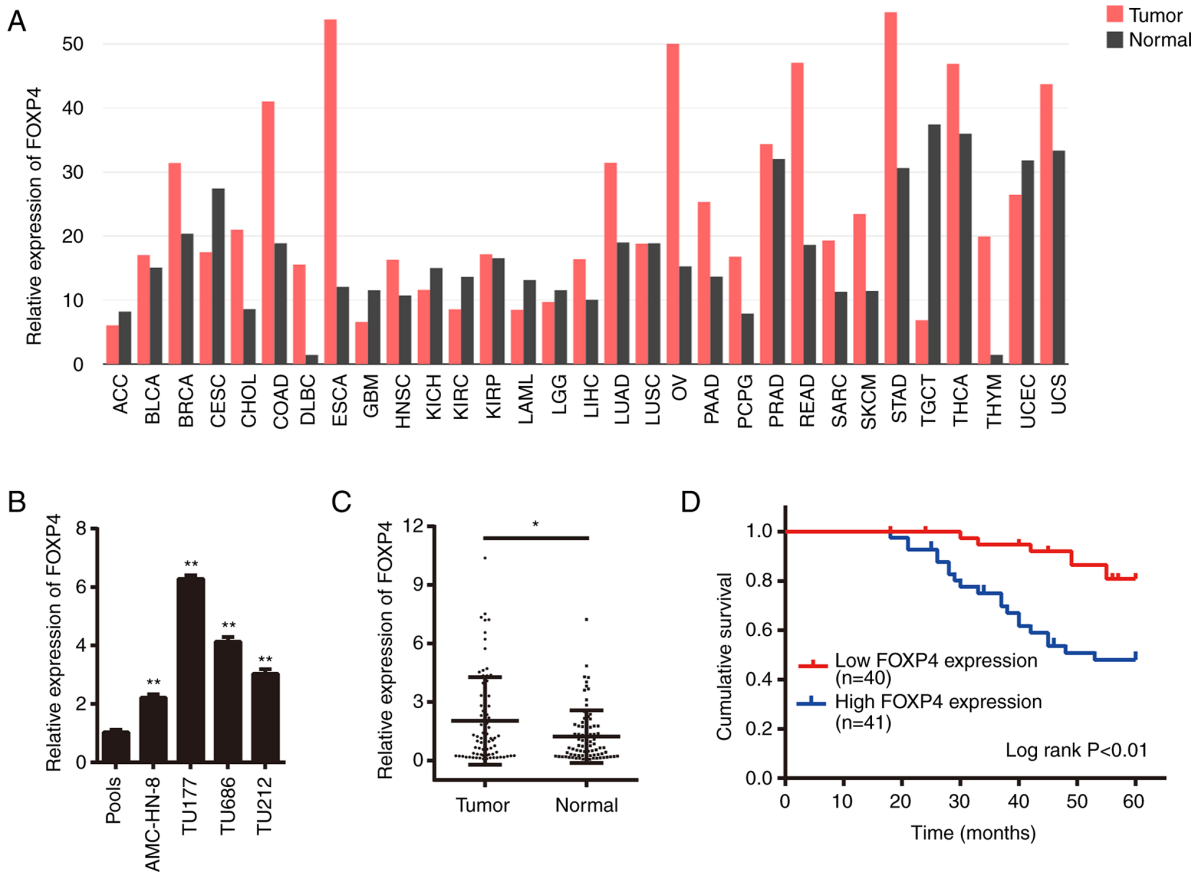

Figure 1. FOXP4 expression is significantly upregulated and closely related with the survival of patients with LSCC. (A) Relative expression of FOXP4 in various tumor types cited from the Gene Expression Profiling Interactive Analysis dataset. (B) FOXP4 expression in laryngeal cancer cell lines detected via RT-qPCR assay. **P<0.01 vs. pools group. (C) FOXP4 expression in 81 pairs of LSCC tissues and adjacent normal tissues detected via RT-qPCR. (D) Overall survival of patients with LSCC according to FOXP4 expression by Kaplan-Meier analysis. *P<0.05 vs. normal group. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower-grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; FOXP4, forkhead box P4; LSCC, laryngeal squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR.

72 h, as siRNA produces short-term inhibition of gene expression. The clone formation assay showed that clone formation was significantly inhibited in the si-FOXP4-1 and si-FOXP4-2 groups compared with the si-NC group, whereas it was significantly increased in the pcDNA3.1-FOXP4 group compared with the pcDNA3.1 group (Fig. 2D). Furthermore, transfection of si-FOXP4 or pcDNA3.1-FOXP4 affected the migratory and invasive abilities of TU177 and AMC-HN-8 cells *in vitro* (Fig. 2E and F).

FOXP4 contributes to EMT process induced by TGF-β1. An increasing number of studies have shown the important role of EMT in regulating tumor invasiveness and metastasis (14-16), thus the effects of FOXP4 on EMT characteristics were further examined in the present study. The phenotype of TU177 cells in the presence of TGF-β1 was observed and it was found that cells exhibited a spindle-shaped morphology characteristic of EMT (Fig. 3A). Subsequently, the expression levels of EMT-related markers and FOXP4 were found to be upregulated in TGF-β1-treated TU177 cells compared with untreated cells, whereas epithelial marker (E-cadherin) was downregulated (Fig. 3B and C). These findings indicated that the TU177 cells displayed EMT-associated characteristics and FOXP4 could be induced by TGF-β1.

Moreover, the role of FOXP4 on the mRNA expression levels of EMT-related markers was explored. It was shown that knockdown of FOXP4 significantly increased E-cadherin and reduced N-cadherin, Vimentin, Twist, Snail and zinc finger E-box-binding homeobox 1 (ZEB1) expression in TU177 cells compared with the si-NC group (Fig. 3D). Meanwhile, the expression of E-cadherin was decreased, and N-cadherin, Vimentin, Twist, Snail and ZEB1 expression levels were upregulated following FOXP4 overexpression in AMC-HN-8 cells compared with the pcDNA3.1 group (Fig. 3E). The expression of E-cadherin in TU177 cell lines was significantly upregulated in the si-FOXP4 groups compared with that in the si-NC group, whereas N-cadherin, Vimentin, Twist and Snail expression levels were significantly downregulated. Besides, the expression of E-cadherin was significantly downregulated in AMC-HN-8 cell lines with FOXP4 overexpression

Table I. Association between the expression of FOXP4 and the clinicopathological features of patients with laryngeal squamous cell carcinoma.

Characteristics	Number of patients	FOXP4 expression		P-value
		Low, n (%)	High, n (%)	
Age, years				0.224
<60	37	21 (56.76)	16 (43.24)	
≥60	44	19 (43.18)	25 (56.82)	
Smoking				0.094
No	33	20 (60.61)	13 (39.39)	
Yes	48	20 (41.67)	28 (58.33)	
Lymph node metastasis				0.027[a]
Negative	51	30 (58.82)	21 (41.18)	
Positive	30	10 (33.33)	20 (66.67)	
TNM stage				0.001[b]
I+II	37	26 (70.27)	11 (29.73)	
III+IV	44	14 (31.82)	30 (68.18)	
Pathological differentiation				0.037[a]
Well/moderate	56	32 (57.14)	24 (42.86)	
Poor	25	8 (32.00)	17 (68.00)	

[a]P<0.05 and [b]P<0.01. FOXP4, forkhead box P4; TNM, tumor node metastasis.

Table II. Univariate and multivariate Cox regression analysis for clinicopathological features associated with prognosis of 81 patients with laryngeal squamous cell carcinoma.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years, <60 vs. ≥60	1.642 (0.752-3.588)	0.214	0.416 (0.144-1.203)	0.106
Smoking, no vs. yes	1.508 (0.677-3.359)	0.314	1.766 (0.764-4.079)	0.183
Lymph node metastasis, negative vs. positive	3.529 (1.629-7.647)	0.001[c]	1.014 (0.368-2.795)	0.978
TNM stage, I+II vs. III+IV	10.768 (3.226-35.945)	0.000[c]	6.295 (1.615-24.538)	0.008[b]
Pathological differentiation, well/moderate vs. poor	5.800 (2.642-12.734)	0.000[c]	4.555 (1.516-13.685)	0.007[b]
FOXP4 expression, low vs. high	3.859 (1.626-9.159)	0.002[c]	2.691 (1.083-6.687)	0.033[a]

[a]P<0.05, [b]P<0.01, [c]P<0.005. FOXP4, forkhead box P4; TNM, tumor node metastasis.

compared with those in the pcDNA3.1-control group, whereas N-cadherin, Vimentin, Twist and Snail expression levels were markedly upregulated (Fig. 3F). Taken together, the aforementioned results indicated that FOXP4 promoted EMT in laryngeal cancer cells.

FOXP4 directly targets LEF-1 and activates Wnt signaling pathway. To further investigate the downstream targets of FOXP4, the Animal TFDB 3.0 database was screened and potential FOXP4-binding elements in the LEF-1 proximal promoter were predicted (17) (Fig. 4A).

First, the expression of LEF-1 in LSCC tissues was detected and it was found that LEF-1 expression was significantly upregulated in tumor tissues compared with the normal tissues (Fig. 4B). In addition, a moderate positive correlation between FOXP4 and LEF-1 expression in head and neck squamous cell carcinoma was predicted using data from GEPIA (Fig. 4C). Similarly, a moderate positive correlation was also confirmed between FOXP4 and LEF-1 in LSCC tissues (Fig. 4D). Furthermore, transfection with si-FOXP4-1 led to the significant downregulation of LEF-1 expression compared with the si-NC group, while transfection with pcDNA3.1-FOXP4 caused significant upregulation of LEF-1 expression compared with the pcDNA3.1 group (Fig. 4E). The ChIP assay confirmed that FOXP4 was directly recruited to the LEF-1 promoter in AMC-HN-8 cells transfected with pcDNA3.1-FOXP4 (Fig. 4F). Consistently, silencing FOXP4 significantly decreased the relative luciferase activity of LEF-1

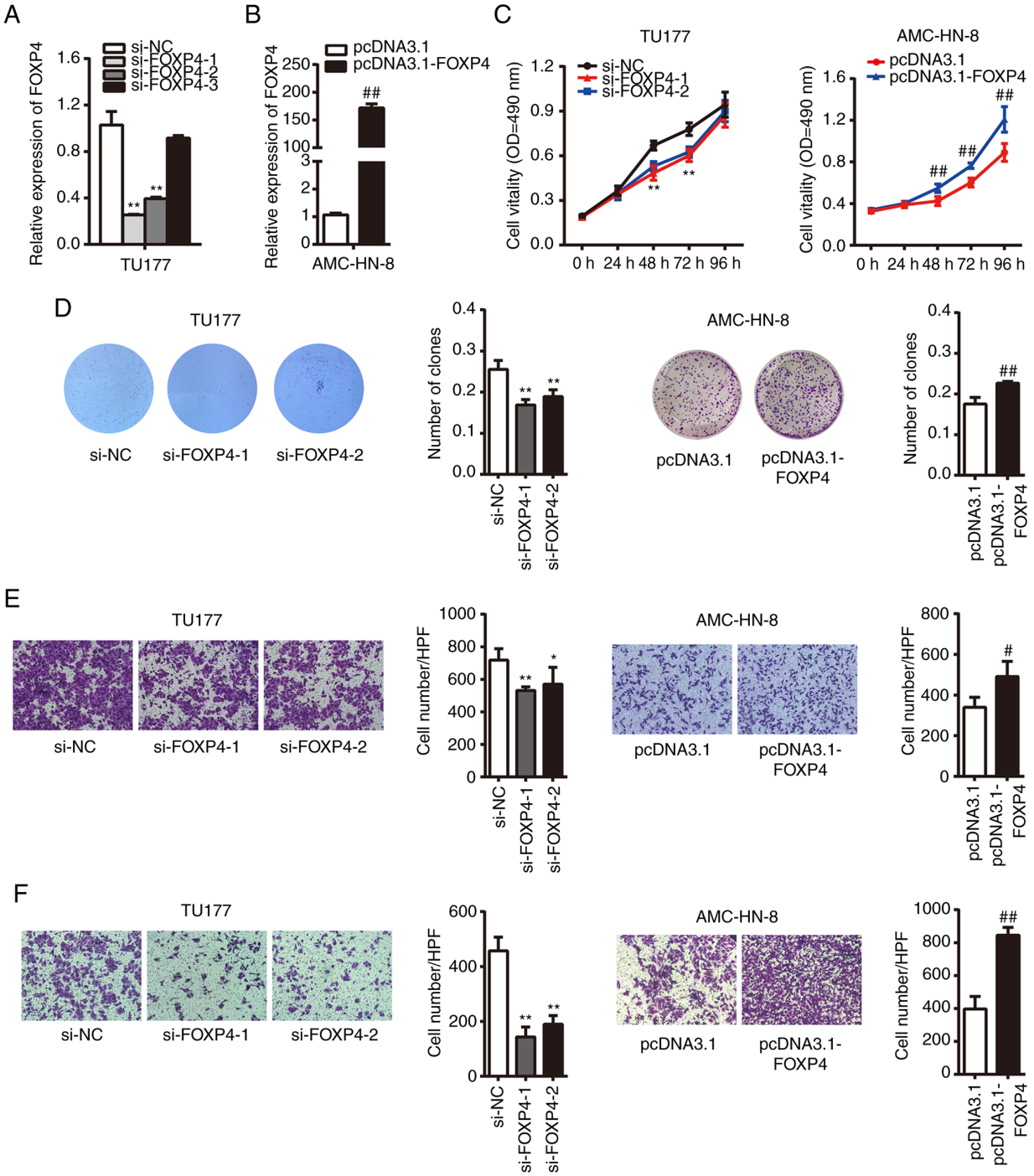

Figure 2. FOXP4 regulates the biological processes of laryngeal cancer cells. The transfection efficiency of (A) siRNAs against FOXP4 and (B) pcDNA3.1-FOXP4, as determined via reverse transcription-quantitative PCR. (C) Cell viability was detected using an MTS assay following FOXP4 knockdown or overexpression. (D) Clone formation assay was conducted following FOXP4 knockdown or overexpression. Transwell (E) migration and (F) invasion assays were performed following FOXP4 knockdown or overexpression. *P<0.05 and **P<0.01 vs. si-NC group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. pcDNA3.1 group. FOXP4, forkhead box P4; si-, small interfering RNA; NC, negative control; HPF, high power field.

promoter in the group that contained the FOXP4-binding site, whereas FOXP4 overexpression had the opposite effects (Fig. 4G). These results indicated that FOXP4 directly targeted and positively regulated LEF-1.

Due to the fact that LEF-1 plays crucial roles in Wnt signaling, it was hypothesized whether FOXP4 could modulate Wnt signaling downstream genes. The results demonstrated that FOXP4 positively regulated the expression levels of G1/S-specific cyclin-D1 (CCND1), JUN, MYC and MMP7 (Fig. 4H), which are well-established downstream target genes of the Wnt pathway (18). Collectively, these data suggested that FOXP4 activates the Wnt signaling pathway.

Discussion

Abnormal gene expression is involved in the malignant biological behavior of LSCC. In the present study, it was found that FOXP4 was significantly upregulated in LSCC tissues

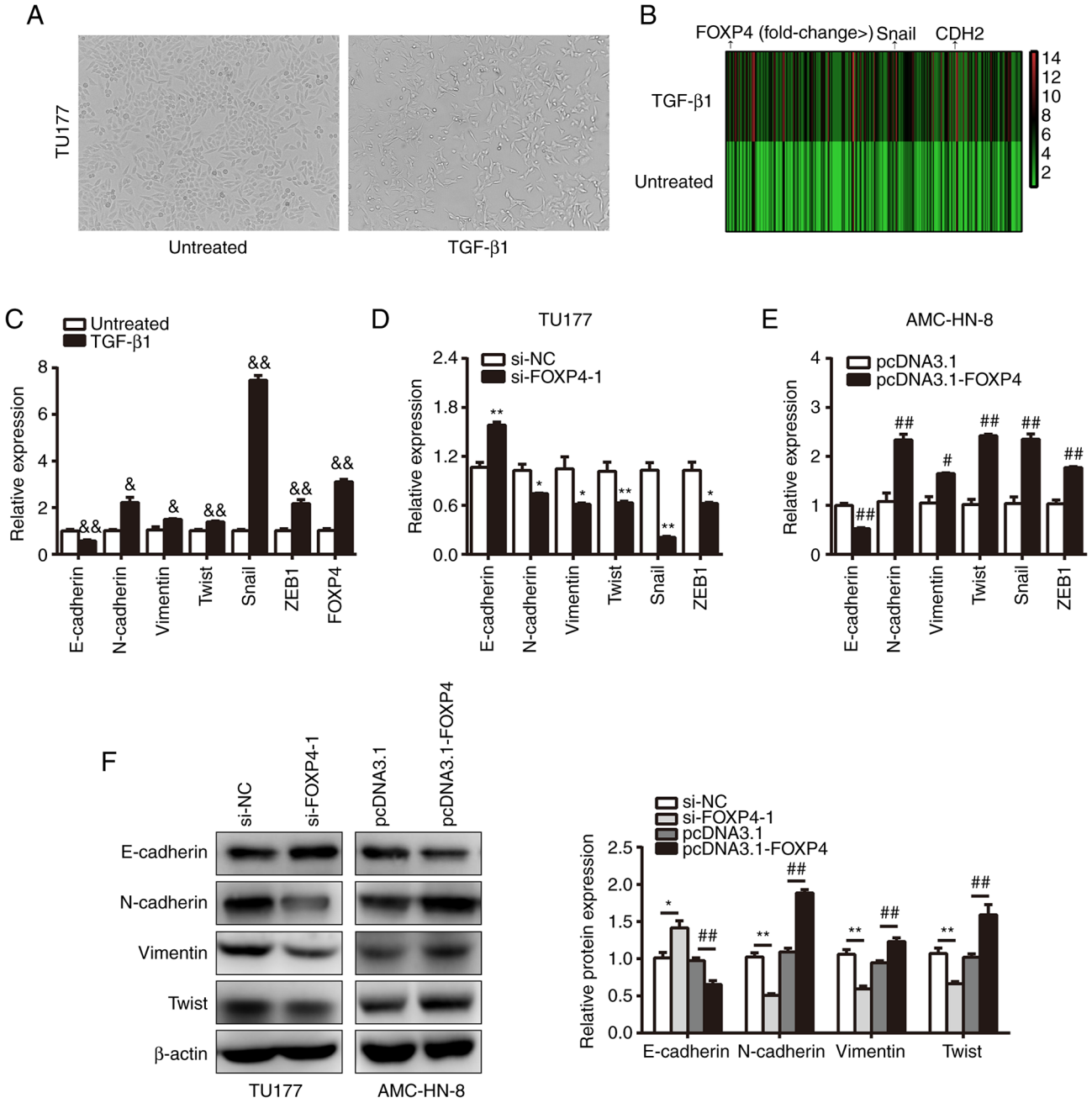

Figure 3. FOXP4 participates in TGF-β1-induced EMT. (A) Cell morphological changes of TU177 cells after TGF-β1 treatment (magnification, x10). (B) Heatmap for genes regulated by TGF-β1. In the heatmap, genes upregulated (>2-fold) by TGF-β1 are shown in red; genes downregulated (>2-fold) by TGF-β1 are shown in green. (C) The expression levels of EMT markers and FOXP4 in TGF-β1-treated TU177 cells compared with untreated cells were determined via RT-qPCR. The expression of EMT markers were measured via RT-qPCR following FOXP4 (D) knockdown or (E) overexpression. (F) The effects of FOXP4 on EMT markers were observed via western blotting. $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. TGF-β1 untreated group; *P<0.05 and **P<0.01 vs. si-NC group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. pcDNA3.1 group. FOXP4, forkhead box P4; TGF-β1, transforming growth factor- β1; EMT, epithelial-mesenchymal transition; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering RNA; NC, negative control; ZEB1, zinc finger E-box-binding homeobox 1.

and laryngeal cancer cell lines and closely associated with the poor survival rate of patients with LSCC. Besides, FOXP4 possessed roles as an oncogene in promoting cell viability, migration, invasion and EMT process, and directly bound to the promoters of LEF-1 to activate Wnt signaling. Thus, this study delineated a molecular mechanism of FOXP4 in facilitating LSCC progression and improves our understanding of this transcription factor.

The human FOX gene family consists of at least 43 members, which are mainly involved in tumorigenesis through gene amplification, retroviral integration, chromosomal translocation and transcriptional regulation (19). At present, an increasing number of studies have revealed that FOX family genes fulfil their roles as transcriptional activators or repressors in cancer initiation, progression and chemoresistance. For example, FOXA1 has been found to occupy the upstream enhancer of the transforming growth factor β-3 proprotein promoter to repress its transcription in castration-resistant prostate cancer (20). FOXA2 can target and simulate the E-cadherin promoter

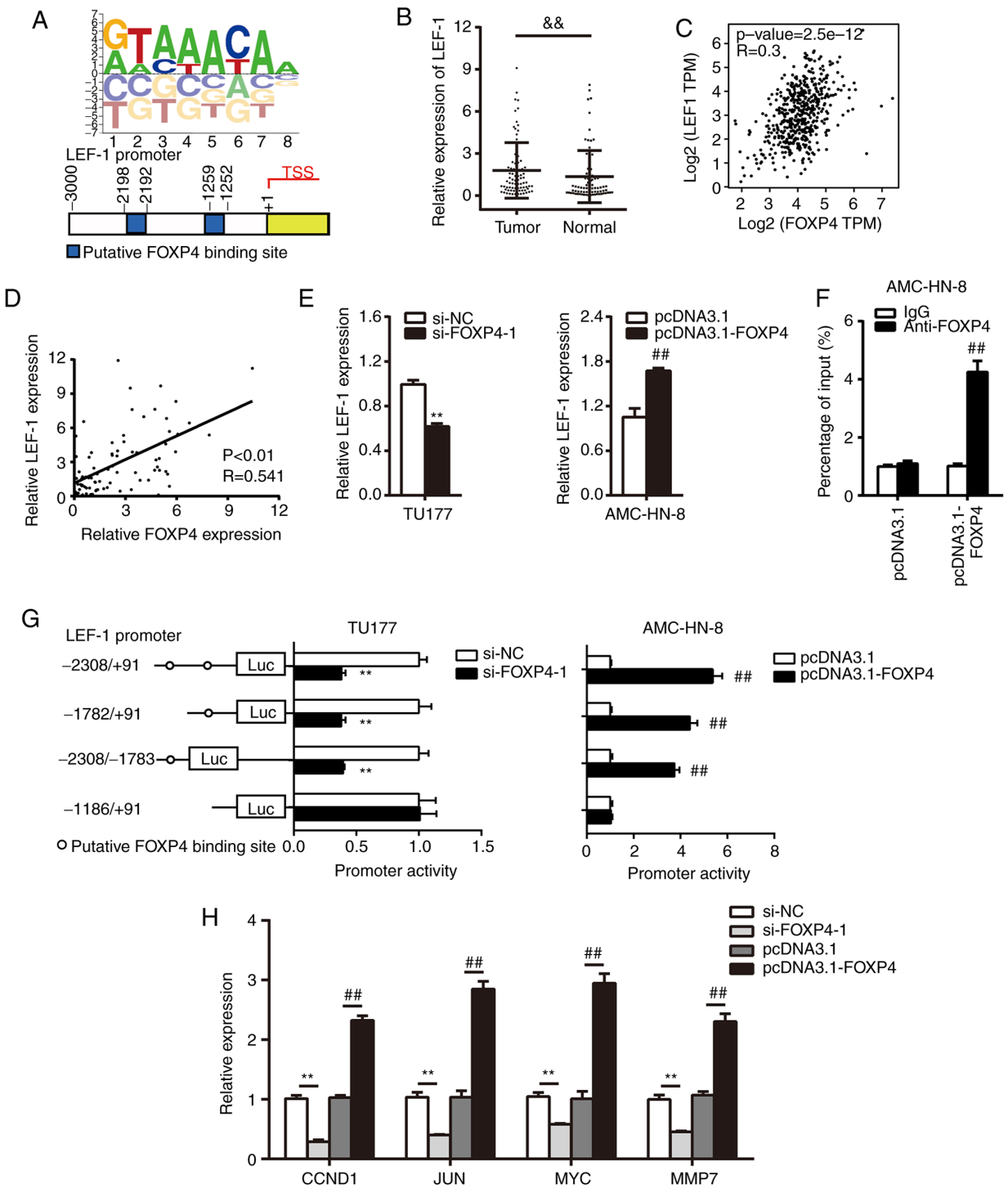

Figure 4. FOXP4 directly upregulates LEF-1 and plays a role in Wnt pathway activation. (A) Map of FOXP4 binding site sequence and schematic illustration of two potential FOXP4 binding sites in the LEF-1 promoter. (B) LEF-1 expression in LSCC tissues and adjacent normal tissues determined via reverse transcription-quantitative PCR. (C) The positive correlation between FOXP4 and LEF-1 predicted by Gene Expression Profiling Interactive Analysis in head and neck squamous cell carcinoma. (D) The correlation between FOXP4 and LEF-1 expression. (E) Relative expression of LEF-1 in FOXP4-knockdown or -overexpression cells. (F) The enrichment of FOXP4 at the promoter region of LEF-1 was determined using a chromatin immunoprecipitation assay. (G) The luciferase activity of the LEF-1 promoter containing or lacking FOXP4-binding elements was assessed using a dual-luciferase reporter assay. (H) Relative expression levels of Wnt signaling-related target genes in FOXP4-knockdown or -overexpression cells. $^{\&\&}$P<0.01 vs. normal group; **P<0.01 vs. si-NC group; $^{\#\#}$P<0.01 vs. pcDNA3.1 group. FOXP4, forkhead box P4; LEF-1, lymphoid enhancer binding factor-1; si-, small interfering RNA; NC, negative control; CCND1, G1/S-specific cyclin-D1.

and recruit transducin-like enhancer protein 3 to the ZEB2 promoter to inhibit ZEB2 expression in breast cancer (21). Furthermore, it has been observed that FOXC2 can target the angiopoietin-2 promoter to regulate its expression in hepatocellular carcinoma cells (22). FOXM1 can directly activate chromosome-associated kinesin KIF4A (KIF4A) gene transcription by binding to the KIF4A promoter in hepatocellular carcinoma (23).

Members of the FOXP family have also been demonstrated to function as oncogenes or tumor suppressors in tumorigenesis and progression. Sheng *et al* (24) reported that FOXP1 was downregulated and contributed to lung adenocarcinoma cell progression via chemokine signaling pathways. Chen *et al* (25) indicated that FOXP2 acted as a novel suppressor in regulating EMT process via the TGFβ/SMAD signaling pathway in breast cancer. Zhang *et al* (26) corroborated that FOXP3 suppressed breast cancer metastasis and directly bound to the promoter of CD44 to inhibit its expression. Similarly, FOXP4 has been demonstrated to promote cell growth and metastasis in breast cancer, hepatocellular carcinoma and oral squamous cell carcinoma (7,8,10). However, to the best of our knowledge, there are no findings concerning the role of FOXP4 in regulating gene transcription in LSCC, thus the present study examined the expression level of FOXP4 and potential regulatory mechanisms in LSCC. It was demonstrated that FOXP4 was upregulated in LSCC tissue and laryngeal cancer cell lines and displayed an oncogenic role in LSCC tumorigenesis.

EMT is a complex molecular process that contributes to tumor progression and metastasis that is characterized by the decreased expression of E-cadherin and increased expression of vimentin and N-cadherin (5,27). Besides, EMT-initiating transcription factors are implicated in inducing the EMT phenotype (28). Most previous studies have demonstrated that TGF-β-induced genes play pivotal roles in EMT to regulate the malignant biological behavior of different cancer types. Cui *et al* (29) reported that MIR155HG induced by TGF-β participated in LSCC progression and EMT by regulating the miR-155-5p/SOX10 axis. Feng *et al* (30) observed that TGF-β-dependent upregulation of FAM83H-AS1 promoted EMT process in esophageal squamous cell carcinoma progression. In the present study, TGF-β1-induced FOXP4 expression increased N-cadherin, Vimentin, Twist, Snail and ZEB1 expression at the mRNA and protein levels in laryngeal cancer cells, which indicated that FOXP4 was a TGF-β-induced transcription factor and promoted EMT process in LSCC.

As a transcriptional factor, FOXP4 is involved in the activation of several genes that have a crucial role in various biological processes for cancer progression (7,8). For example, Ma and Zhang (7) found that FOXP4 promoted EMT through transcriptionally activating Snail expression in breast cancer. Another study revealed that FOXP4 was significantly upregulated and facilitated EMT by transcriptionally regulating Slug in hepatocellular carcinoma (8). The current study verified that FOXP4 directly targeted the LEF-1 promoter and regulated its transcriptional activity, as confirmed via ChIP and luciferase reporter assays. LEF-1 is a member of the LEF/TCF transcription factor family and is a well-known binding partner of β-catenin, which promotes the activation of Wnt signaling (18). Whether FOXP4 exerted its carcinogenic effect via regulating Wnt signaling was further examined in the present study by measuring the expression levels of the Wnt/β-catenin signaling-related target genes. It was found that FOXP4 positively regulated CCND1, JUN, MYC and MMP7 expression, illustrating that FOXP4 may mediate tumorigenesis and progression of LSCC via Wnt signaling.

However, there were several limitations of the present study. First, the detailed mechanism of FOXP4 in LSCC progression requires further investigation. Furthermore, *in vivo* assays are necessary to verify the present conclusions, which were not performed in this study.

In conclusion, this study demonstrated that FOXP4 was induced by TGF-β and functioned as an oncogene in LSCC. Besides, FOXP4 was associated with tumor progression and poor prognosis. FOXP4 was demonstrated to regulate the EMT process, and could directly bind to the LEF-1 promoter to activate Wnt signaling. Thus, FOXP4 may be a potential strategy for inhibiting LSCC progression and metastasis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ designed the study and revised the manuscript. JS and JW wrote the manuscript. JS, JW, HC, SL, XH, LL, GW and ML recruited the patients, performed the experiments, analyzed the data and drafted the paper. YZ and JS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University (approval no. 2020k-1147; Shijiazhuang, China). Patients provided signed informed consent prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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