

Label-free quantitative proteomic analysis identifies CTNNB1 as a direct target of FOXP3 in gastric cancer cells

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Abstract. Forkhead box protein 3 (FOXP3) is expressed in numerous types of tumor cell and is associated with tumor progression and prognosis. A previous study reported that FOXP3 inhibited cellular proliferation and induced apoptosis of gastric cancer (GC) cells by activating the apoptosis signaling pathway. In the present study, label-free quantitative proteomic analysis and chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) was performed to investigate the mechanism by which the anticancer role of FOXP3 was mediated and the proteins that with which it may interact. Label-free quantitative proteomic analysis was used to screen for proteins differentially expressed between FOXP3-overexpressing GC (AF) and vector (ANC) cells. Catenin β 1 (CTNNB1) was one of the proteins that exhibited the greatest difference between AF and ANC among 3,313 proteins identified by liquid chromatography with tandem mass spectrometry analysis. The expression of CTNNB1 was evaluated by reverse transcription-quantitative PCR and western blotting. The association between FOXP3 and CTNNB1 was confirmed by ChIP-PCR in AGS cells. The changes in expression of epithelial-mesenchymal transition-associated proteins were analyzed by western blotting. The level of FOXP3 expression was positively associated with CTNNB1 and E-cadherin expression, but not with vimentin and N-cadherin expression. FOXP3 positively regulates CTNNB1 and binds to it directly. Along with the upregulation of glycogen synthase kinase 3 β (GSK3 β), which was also a protein whose expression was found to change significantly in proteomic analysis and has a

key role in the Wnt pathway. This association is an attractive and novel hypothesis for the mechanism by which FOXP3 inhibits the invasion and metastasis of GC cells.

Introduction

Gastric cancer (GC) is a malignant tumor of the gastrointestinal tract that is common worldwide (1). Although the incidence of GC has decreased since the 1990s in the majority of developed countries, outcomes in patients with advanced GC remain poor, and the 5-year survival rate ranges between 4 and 20% for patients with surgically resected tumors (2). Invasion and metastasis of GC are the two main reasons for its poor prognosis. Forkhead box protein 3 (FOXP3) is a transcription factor that is necessary for the induction of the immunosuppressive functions in regulatory T cells (3). Previous studies reported the expression of FOXP3 has been observed in a number of types of tumor cell (4,5). The expression of FOXP3 in different tumor cells may drive different functions. In a previous study, high FOXP3 expression in GC cells was found to predict longer survival times (6). FOXP3 was also observed to inhibit proliferation and induce apoptosis in GC cells by activating the apoptotic signaling pathway (7). FOXP3 is reported to negatively regulate nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and thus has a tumor suppressor role in GC (8). However, the mechanisms by which FOXP3 acts as a tumor suppressor remain largely unknown.

GC is a polygenic disease linked with the transcription and expression of multiple oncogenes and tumor suppressor genes, which finally express in the form of protein. Label-free quantitative proteomic analysis is a newly emerged, efficient, powerful and cost-effective approach for comparing various proteins from different samples (9). Label-free proteomic analysis can screen differential protein expression with a high efficiency (10). In the present study, cells were digested by the filter-aided sample preparation (FASP) procedure, to obtain purer peptides and higher image quality. FOXP3-overexpressing AGS cells were used as the experimental group and empty vector-transfected AGS cells as the control group. A total of 3,313 protein groups were quantified under highly rigorous criteria with a false discovery rate of <1% for peptide and protein groups. Of these proteins,

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276 exhibited differences in expression that were statistically significant between FOXP3-overexpressing and control cells.

In the present study, label-free proteomic analysis was performed to investigate the molecules through which FOXP3 mediates its anticancer role and to obtain a better understanding of its mechanism of action in GC.

Materials and methods

Cell lines, reagents. Human GC cells AGS were purchased from the Chinese Academy of Science (Shanghai, China). The AGS cells were cultivated in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in 5% CO₂.

Transfection. The lentivirus of FOXP3 overexpression and its vehicle were purchased from Genepharma (Shanghai GenePharma Co., Ltd., Shanghai, China). The AGS cells were grown to 70–80% confluence in six well plates. The AGS cells were infected with a multiplicity of infection (MOI) of 50 to generate FOXP3-overexpression (referred to as AF). Cells infected with an empty vector served as control cells (referred to as ANC). A total of 2.5 µg/ml puromycin was added to AF cells 48 h after transfection to select cells stably expressing FOXP3. Subsequent experiments were conducted immediately following the selection.

Protein preparation and label-free proteomic analysis. Cells were digested using the FASP procedure, as described previously (11). Briefly, 10⁷ AGS cells were washed with phosphate buffered saline (PBS) three times to remove, then lysed using SDT buffer (containing 4% SDS (m/w), 100 mM DTT and 100 mM Tris, pH 7.6). The lysate was incubated at 95°C for 5 min and then centrifuged at 15,000 x g for 10 min at 4°C, and the supernatant was used for proteomics sample preparation. Label-free proteomic analysis was performed as previously described (9). The reverse phase high-performance liquid chromatography (HPLC) separation was achieved using the EASY-nLC1000 HPLC system (Thermo Fisher Scientific, Inc.) using a self-packed column (75 µm x 150 mm; 3 µm ReproSil-Pur C18 beads, 120 Å; Dr. Maisch HPLC GmbH, Ammerbuch, Germany) at a flow rate of 300 nl/min using 240 min gradients. The full mass was scanned in the Orbitrap analyzer with R=70,000 (defined at m/z 200), and the subsequent MS/MS analyses were performed with R=17,500. Proteins were identified by searching the MS and MS/MS data for the peptides against a decoy version of the International Protein Index human or rat database (version 3.87, 91464 protein sequences; European Bioinformatics Institute, Hinxton, Cambridge, UK). Trypsin/P was selected as the digestive enzyme with two potential missed cleavages. Protein abundance was calculated according to the normalized spectral protein intensity (LFQ intensity).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using commercial RNA isolation kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol, and 2 µg RNA was reverse transcribed to cDNA using oligodT primers with the

Primer Script™ RT Reagent (Takara Biotechnology Co., Ltd.). The target genes FOXP3, catenin β1 (CTNNB1), E-cadherin, N-cadherin, and vimentin, and the internal control gene GAPDH were then amplified by qPCR. The RT-qPCR was performed in a total volume of 10 µl, with 2X SYBR® Green Mix (Takara Biotechnology Co., Ltd.) in the ABI PRISM 7500 system (PerkinElmer, Inc., Waltham, MA, USA). All samples were run in triplicate. The amplification reaction was initiated by denaturing DNA at 95°C for 5 min, followed by 40 cycles of template denaturing at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. The comparative C_q method (2^{-ΔΔC_q} method) was used for RT-qPCR data analysis (12).

The primers used were as follows: FOXP3 forward, 5'-AAG CAGCACTACATTGACCTGAAA-3' and reverse, 5'-GGT CTCCCCAAGCATCACTC-3'; CTNNB1 forward, 5'-TGG TGACAGGGAAGACATCA-3' and reverse, 5'-CCATAGTGA AGGCGAACTGC-3'; E-cadherin forward, 5'-GGTCTCTCT CACCACCTCCA-3' and reverse, 5'-CCTCGGACACTTCCA CTCTC-3'; N-cadherin forward, 5'-CGTGAAGGTTTGCCA GTGT-3' and reverse, 5'-CAGCACAAGGATAAGCAGGA-3'; vimentin forward, 5'-GTACCGGAGACAGGTGCAGT-3' and reverse, 5'-AACGGCAAAGTTCTCTTCCA-3'; and GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGGA-3'.

Western blot analysis. Cells were collected and proteins were extracted with radioimmunoprecipitation assay lysis buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The protein concentration was determined by the BCA protein assay (Shanghai Yeasen Biotech Co., Ltd., Shanghai, China). Protein samples (40 µg per lane) were separated by 10% SDS-PAGE and then electro-transferred onto nitrocellulose membranes for 90 min. The membranes were blocked for 1 h with 5% skimmed milk at room temperature and incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies. Primary antibodies used were as follows: Specific to FOXP3 (cat no. 236A/E7; 1:500; anti-mouse; Abcam, Cambridge, MA, USA), CTNNB1 (cat no. 8480; 1:1,000; anti-rabbit), E-cadherin (cat no. 3195; 1:1,000; anti-rabbit), N-cadherin (cat no. 13116; 1:1,000; anti-rabbit), Vimentin (cat no. 5741; 1:1,000; anti-rabbit), GSK3β (cat no. 12456; 1:1,000; anti-rabbit) and GAPDH (cat no. 2118; 1:5,000; anti-mouse) (all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA). Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit/mouse immunoglobulin G (cat no. 111-035-003/115-035-003; 1:5,000; Jackson Laboratory, Bar Harbor, ME, USA). The reactions were visualized using enhanced chemiluminescence kit (Merck KGaA, Darmstadt, Germany) and a gel imaging system.

Chromatin immunoprecipitation followed by PCR (ChIP-PCR). ChIP was performed in native conditions. In brief, cells at a concentration of 2x10⁶/ml were treated with 1% formaldehyde for 10 min at room temperature. Glycine (1.25 M) was then added and the samples were incubated for 5 min at room temperature. The cells were washed twice with cold PBS and pelleted. Each pellet was resuspended in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM DTT and 1 mM

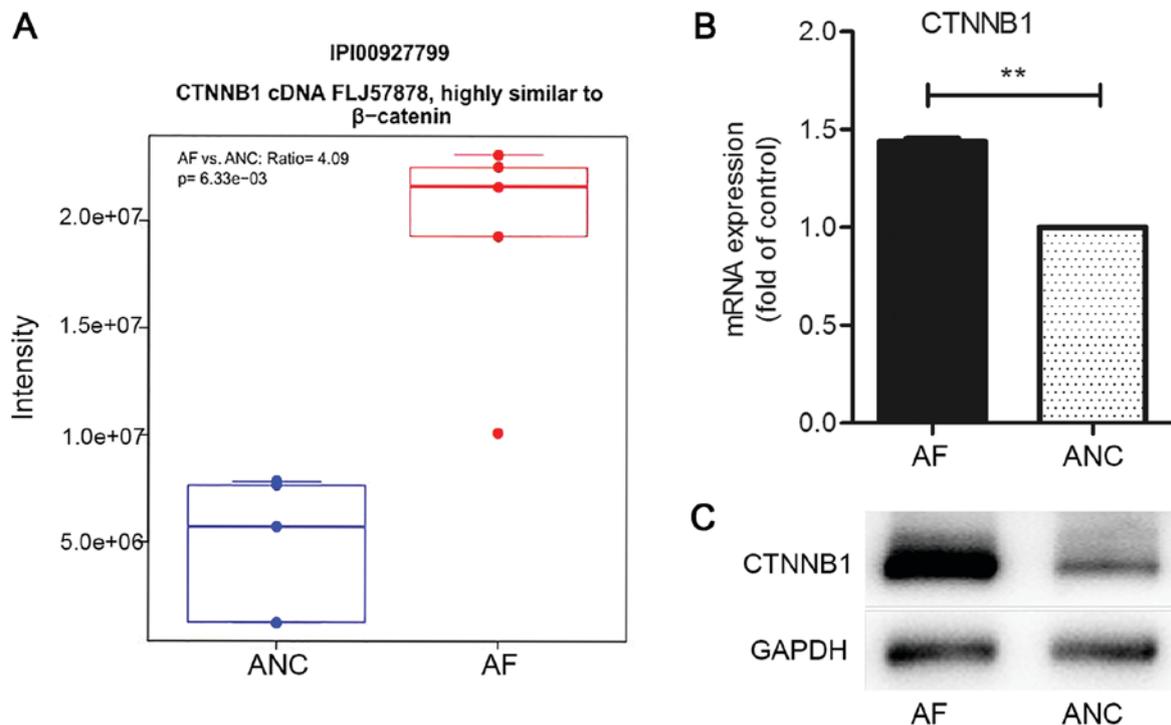


Figure 1. FOXP3 is associated with the expression of CTNNB1. (A) Proteomic analysis showing box and whisker plots for the label-free quantification intensity of CTNNB1 ($P=0.006$; fold change, 4.09). (B) RT-qPCR of the mRNA expression level of CTNNB1. (C) Western blot analysis of CTNNB1. $**P<0.01$. FOXP3, forkhead box protein 3; CTNNB1, catenin β 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

phenylmethylsulfonyl fluoride/cocktail), incubated on ice with vortexing for 10 min, and the lysate was obtained by centrifugation at 4°C with 13,000 x g for 10 min. The majority of the DNA fragments were sheared by sonication on ice to a length of 200-500 bp. Antibodies specific to FOXP3 (cat no. ab2481, 1:50, Abcam), RNA pol2 (cat no. sc-899, 1:100, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and protein G beads were added and samples were incubated for 4 h at 4°C. The samples were washed once in a low-salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), once in a high-salt wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), once in a LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and once in a TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The beads were then resuspended in lysis buffer and treated with proteinase K at 45°C for 45 min. Co-precipitated DNAs were purified with phenol-chloroform to eliminate SDS sediment. The effectiveness of CHIP was detected using PCR. The PCR was performed to verify whether these DNA fragments were between 200-500 bp using the 2X Hieff™ HotStart PCR Master Mix (Yeasen Biotechnology Co., Ltd., Shanghai, China) with DNA ladder (cat no. D0107, Beyotime Institute of Biotechnology, Haimen, China) as the marker. The sequences of primers were: FOXP3 forward, 5'-AAGCAGCACTACATT GACCTGAAA-3'; FOXP3 reverse, 5'-GGTCTCCCAAG CATCACTC-3'; GAPDH forward, 5'-ATGGGGCGCACG GCGGGAATG-3'; GAPDH reverse 5'-CTCCTTGGGCGC TTCGCC-3'. The thermocycling conditions were 35 cycles of 95°C for 30 sec for denaturation, 55°C for 30 sec for annealing and 72°C for 30 sec for elongation. PCR products were loaded onto a 1% agarose gel, and the bands were visualized using the

Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data were analyzed using the SPSS 20.0 statistical program (IBM Corp., Armonk, NY, USA). Values are expressed as fold change or mean \pm standard error of the mean. Unpaired Student's t-tests were used for comparisons between two means. $P<0.05$ was considered to indicate statistical significance.

Results

Label-free quantitative proteomic analysis of AF and ANC cells. In an attempt to identify changes in proteins, the differential protein expression between the AF and the ANC cells was monitored. To ensure the reproducibility of the results, five biological replicates were performed for proteomic analysis. The high homogeneity of AF and ANC resulted in highly accurate liquid chromatography with tandem mass spectroscopy identification results. Of a total of 3,313 proteins, the expression of 276 differed significantly ($P<0.05$, using a \log_2 fold-change >2 as the cut-off). The expression of CTNNB1 in AF cells was 4.09-fold higher than that in the control cells (Fig. 1A). The level of mRNA expression of CTNNB1 in AF and ANC was verified by RT-qPCR (Fig. 1B). The level of expression of the protein was determined by western blot analysis (Fig. 1C).

Expression of epithelial-mesenchymal transition (EMT)-associated proteins does not differ significantly between AF and ANC. The results of RT-qPCR and western blot analysis indicated that levels of the epithelial phenotype

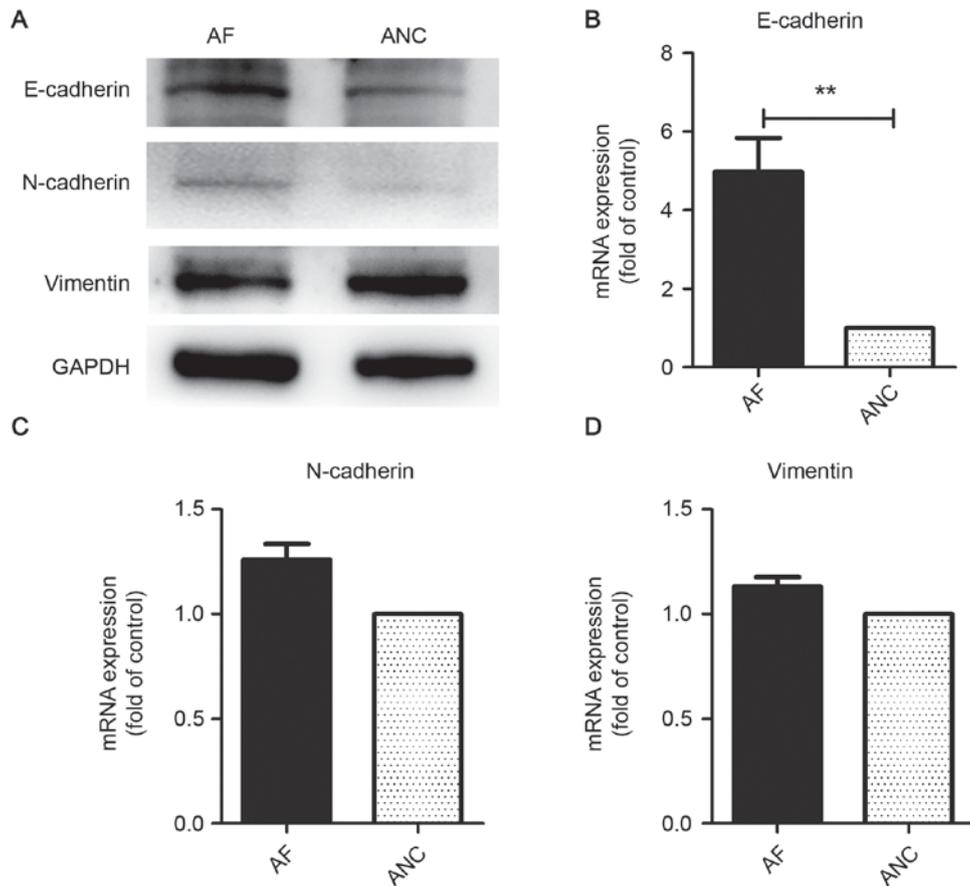


Figure 2. Expression levels of E-cadherin, N-cadherin and vimentin. (A) E-cadherin expression was upregulated in AF cells, but that of vimentin or N-cadherin were not significantly different. (B-D) The results of reverse transcription-quantitative polymerase chain reaction for (B) E-cadherin, (C) N-cadherin and (D) vimentin match those of the western blot. ** $P < 0.01$. AF, cells overexpressing FOXP3; ANC, cells transfected with an empty vector.

protein E-cadherin and CTNNB1 were significantly elevated in AF, but those of the mesenchymal phenotype proteins vimentin and N-cadherin were not (Fig. 2).

Glycogen synthase kinase 3- β (GSK3 β) is upregulated in AF cells. GSK3 β is a key member of the destruction complex that can degrade the CTNNB1 in cytoplasm rapidly (13). Cytoplasmic levels of CTNNB1 are tightly controlled by GSK3 β and the degradation of CTNNB1 in cytoplasm could inhibit the Wnt pathway (13). The expression of GSK3 β in AF cells was found to be 1.49-fold higher than in the control cells. (Fig. 3A) The level of the protein was determined by western blot analysis (Fig. 3B).

FOXP3 interacts with CTNNB1 in AGS cells. The observation that the protein level of CTNNB1 was affected by FOXP3 has been supported by label-free proteomic analysis, and indicates that FOXP3 may modulate the former to a certain degree. To investigate whether FOXP3 affected CTNNB1 by binding to it, a ChIP-PCR assay was performed. ChIP-PCR assays revealed that FOXP3 could bind directly to the promoter region of CTNNB1 in AGS cells (Fig. 4). There were three regions to which FOXP3 could bind. The specific FOXP3 binding positions were located between -1,502 and -1,251 bp, -1,002 and -751 bp, and -751 and 500 bp, starting from the transcription site of the CTNNB1 gene promoter. Data from ChIP-PCR

indicated that FOXP3 could control CTNNB1 directly, and thus affect the downstream pathways.

Discussion

In summary, the results of the present study revealed a novel function of FOXP3, acting as a tumor suppressor through interaction with CTNNB1. In a previous study, FOXP3-positive staining was found to associate with a favorable prognosis in patients with GC, and that upregulation of the FOXP3 gene inhibits GC cell growth *in vitro* and *in vivo* (6). Several mechanisms, including the activation of the apoptotic signaling pathway and inhibition of NF- κ B activity, have been implicated in the anticancer activity of FOXP3 in GC (7,8).

Label-free quantitative proteomic analysis identified CTNNB1 as a novel FOXP3-interacting partner. Consistent with former results, the epithelial phenotype E-cadherin and CTNNB1 were upregulated in FOXP3-overexpressing AGS cells. EMT occurs due to the induction of transcription factors, which alter gene expression to promote the loss of cell-cell adhesion, leading to a shift in cytoskeletal dynamics and a change from epithelial morphology and physiology to the mesenchymal phenotype (14). Cells lose their epithelial characteristics, instead gaining an invasive and migratory mesenchymal phenotype, allowing these cells to leave the tissue parenchyma and enter the systemic

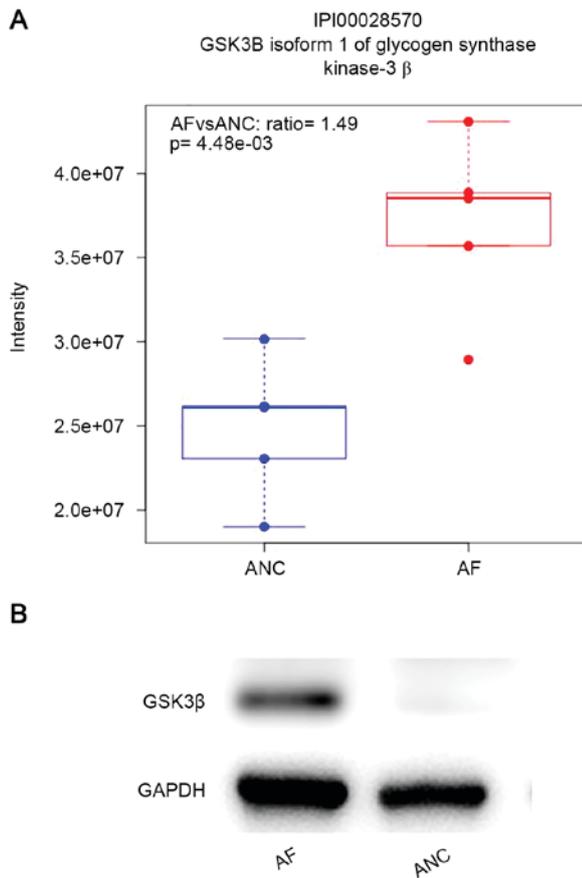


Figure 3. GSK3 β levels in ANC and AF cells. (A) Proteomic analysis showing the label-free quantification intensity of GSK3 β ($P=0.004$; fold change, 1.49). (B) Western blot analysis confirmed the upregulation of GSK3 β . GSK3 β , glycogen synthase kinase 3 β ; AF, cells overexpressing forkhead box protein 3; ANC, cells transfected with an empty vector.

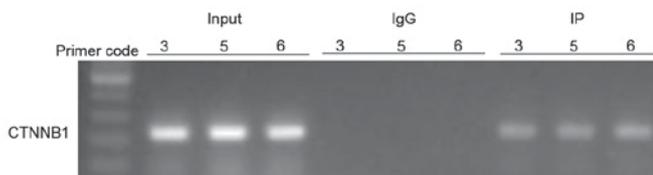


Figure 4. Chromatin immunoprecipitation-polymerase chain reaction verification of FOXP3 binding sites. FOXP3 specifically bound to positions located between -1,502 and -1,251 bp (primer no. 3); -1,002 and -751 bp (primer no. 5); -751 and 500 bp (primer no. 6) from the start site of the CTNNB1 gene promoter. Input, antigen protein used as positive control; IgG, immunoglobulin G negative control; IP, product of immunoprecipitation; FOXP3, forkhead box protein 3; CTNNB1, catenin β 1.

circulation during cancer metastasis. One hallmark of EMT is the dissociation of the E-cadherin-CTNNB1- α -catenin complex from the membrane (15). Thus, the loss of expression of E-cadherin is always accompanied by the loss of expression of CTNNB1 at the membrane. The mesenchymal phenotype indicators vimentin and N-cadherin are always upregulated in EMT.

Given that the role of FOXP3 in EMT has not been investigated previously, the present study measured the mRNA and protein levels of mesenchymal phenotype indicators vimentin and N-cadherin to determine whether

FOXP3 could inhibit EMT. However, the results observed were not expected. Vimentin and N-cadherin were not downregulated in FOXP3-overexpressing cells, indicating that FOXP3 does not mediate its anticancer effect through a mesenchymal-to-epithelial transition. However, using ChIP-PCR, it was identified that FOXP3 could bind directly to the promoter region of CTNNB1. There are three binding domains, located between -1,502 and -1,251 bp, -1,002 and -751 bp, and -751 and -500 bp. These results indicate that FOXP3 upregulates the expression of CTNNB1 in AGS cells.

CTNNB1, commonly known as β -catenin, was first identified as an adhesion molecule by Imhof *et al* (16) in the 1980s. Interest in this molecule increased when it was identified that CTNNB1 is important in the initiation and metastasis of cancer (17). CTNNB1 is a dual-function protein: When there is no Wnt signaling, CTNNB1 dynamically links E-cadherin and α -catenin at the plasma membrane (18). Adhesion to the basement membrane and to adjacent cells is critical for maintaining the epithelial phenotype (19). In the absence of Wnt signaling, CTNNB1 is rapidly degraded by a destruction complex consisting of adenoma polyposis coli, axin, casein kinase and GSK3 β (13). Thus, the concentration of CTNNB1 is maintained at the appropriate level in the cytoplasm. By contrast, in the Wnt signaling pathway the GSK3 β -dependent phosphorylation of CTNNB1 is inhibited; this results in an accumulation of CTNNB1 in the nucleus, where it functions as a transcriptional activator in conjunction with lymphoid enhancer factor/T-cell factor DNA binding proteins and induces EMT (20,21). The signaling function of CTNNB1 is regulated principally through the alteration of its stability in the cytoplasm.

The present label-free quantitative proteomic analysis also identified GSK3 β as a significantly altered protein in AF cells. The expression of GSK3 β was 1.49-fold higher in AF than in APC cells. Since GSK3 β could catalyze the phosphorylation of serine or threonine residues on CTNNB1 substrates, the upregulation of GSK3 β may promote the degradation of CTNNB1 in the cytoplasm and inhibit the Wnt pathway (18), which, together with the increase in CTNNB1 expression on the membrane, could strengthen the E-cadherin-CTNNB1 complex, making it difficult for the GC cells to move.

In summary, proteomic analysis revealed that CTNNB1 levels were significantly upregulated in FOXP3-overexpressing AGS cells. ChIP-PCR revealed that FOXP3 could bind directly to the promoter region of CTNNB1, which means that FOXP3 could directly regulate CTNNB1. These results provide novel information concerning the anticancer mechanism of FOXP3. Further research is required to investigate the possible pathways in which CTNNB1 is involved with FOXP3.

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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

All authors contributed to the conception, design and completion of the present study. DYP and XQZ contributed equally to study design, experimental work and manuscript preparation. GFM designed the study, JG and HZ performed the proteomic experiments, QM and NL analyzed the data and LLX and SYC contributed to refining the study design.

Ethics approval and consent to publish

No human participants, human data or human tissue were involved in the present study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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