Anoctamin5 regulates cell migration and invasion in thyroid cancer

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Received June 9, 2017; Accepted August 25, 2017

DOI: 10.3892/ijo.2017.4113

Abstract. Anoctamin/*TMEM16* family members have recently been identified as novel calcium-activated chloride channels, and dysregulation of many family members participates in tumorigenesis and progression. However, the exact role of anoctamin5 (*ANO5*), one member of this family, in thyroid cancer is still not clarified. In this study, we firstly found that the expression levels of *ANO5* was significantly downregulated in thyroid cancer compared to adjacent normal tissue by mining the public GEO database. Subsequently, we further demonstrated that the expression levels of *ANO5* was significantly downregulated in 69.5% (57/82) clinical thyroid cancer tissues using real-time PCR assay. Moreover, western blot assay also showed that *ANO5* was downregulated in papillary thyroid cancer and follicular thyroid cancer compared to adjacent noncancerous tissues. Furthermore, some biological and functional *in vitro* experiments proved that *ANO5* knockdown promotes thyroid cancer cell migration and invasion but overexpression of *ANO5* inhibits these phenotypes. By analyzing gene set enrichment, we found that lower *ANO5* expression was positively associated with *JAK/STAT3* signaling pathway. Collectively downregulation of *ANO5* promotes thyroid cancer cell migration and invasion by affecting *JAK/STAT3* pathway.

Introduction

Papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) are the most common types of thyroid cancer, accounting for 94% of all cases (1). Most of them are differentiated and have a good prognosis with a 10-year survival rate of >92% (2). But among clinical staging criteria, metastatic lymphadenopathy is one of the best predictors of a poor prognosis, recurrence and motility (3-5) as it likely reflects aggressive primary tumor biology (6,7) (seer.cancer.gov/statfacts/html/oralcav.html). So exploring the mechanism of lymph node metastasis will deepen our understanding of malignant characteristics of some differentiated thyroid cancer.

Anoctamin family (*ANO*, also known as *TMEM16*) contains 10 members which are identified as putative intracellular calcium activated chloride channels (8,9). It has been reported that some members of anoctamin family are overexpressed in cancer (10), moreover, overexpression of *ANO1* and *ANO6* can increase cancer cell migration (11,12). Anoctamin5 (*ANO5*), also referred to as *TMEM16E*, is one member of the anoctamin family. Somatic mutation or microdeletion of *ANO5* usually results in muscular dystrophy (13-18), but its exact role in tumorigenesis and cancer progression is still not clear.

We first evaluated the expression profile of this family in thyroid cancer by mining the public GEO database. We discovered and proved the downregulation of *ANO5* expression in thyroid cancer. Thereafter, we revealed that downregulation of *ANO5* is negatively associated with lymph node metastasis and inhibition of *ANO5* promotes the migration and invasion of thyroid cancer cells. In addition, we also found that lower *ANO5* expression was positively associated with *JAK/STAT3* pathway which is well-known to be activated during cancer metastasis (19,20). The present results provided novel insight into *ANO5*'s function in thyroid cancer metastasis.

Materials and methods

Tissue specimens and cell lines. Thyroid cancer tissue samples used in this study were harvested from Shanghai Tenth People's Hospital between November, 2013 and December, 2015. Written informed consent from all patients was obtained. Thyroid cancer cell lines TPC-1 and FTC-133 were obtained by Dr Lei Ye in Rui-Jin Hospital. TPC-1 cells were maintained in RPMI-1640 medium and FTC-133 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented

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Key words: anoctamin5, thyroid cancer, migration, invasion

with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA) 100 U/ml penicillin, and 100 μg/ml streptomycin.

Antibodies and reagents. The target antibodies were purchased from Abcam.

RNA extraction and real-time PCR. Total RNA was extracted with TRIzol® (Invitrogen, Garlsbad, CA, USA), following the manufacturer's protocol. The OD260/OD280 ratio of RNA ranged from 1.8 to 2.0. Reverse transcription was performed in 20 μl reaction volume with 2 μg of RNA using M-MLV reverse transcriptase kit (Takara, Otsu, Japan). Quantitative real-time PCR was carried out using ABI 7900 Detection system with the SYBR Premix Ex Taq™ (Takara). Primer sequences specific to 38 genes and housekeeping gene GADPH are listed in Table I.

Protein extraction and western blot analysis. Total cellular proteins were extracted using cell lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 10% 2-mercaptoethanol, and protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Then protein concentration was determined using the BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein (30 μg) was subjected to electrophoresis by SDS-PAGE on the 10% gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin (BSA) and 0.1% Tween-20 in PBS for 2 h at room temperature. After incubation with the appropriate primary antibody overnight at 4˚C with anti-*ANO5* (1:500), anti-*STAT3* (1:1,000), anti-p727-*STAT3* (1:1,000) (all from Abcam), and anti-*GAPDH* (1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies, membranes were washed and incubated with the IRDye 800CW secondary antibodies for 1 h at room temperature. The labeled protein bands were detected using the Odyssey Infrared Imaging system (Li-COR Biosciences, Lincoln, NE, USA). *GADPH* was used as a loading control.

Construction of ANO5 expression vectors. Human full-length *ANO5* cDNA (GenBank accession no. NM_001142649.1) was acquired from normal thyroid tissues. Primers for PCR amplification were designed as follows: forward, 5'-ATA TCT AGA ATG GGC GAC CCG GAT CTC CTG GAA G-3' and reverse, 5'-ACG CGG CCG CTT AGA GTG TTG ATT TAG CCA GCT G-3'. The PCR product was subcloned into the pCDH vector (Systembioscience, Inc.) and verified by restriction digestion and DNA sequencing.

Lentivirus production. All recombinant lentiviruses were produced by transfecting HEK293T cells according to standard protocols. In brief, sub-confluent HEK293T cells were co-transfected with 4 μg pCDH-*ANO5* plasmid vector, 3 μg PLP1, 3 μg PLP2 and 2 μg PLP-VSVG in 50 μl Lipofectamine 2000. The medium was changed after 16 h and supernatant was harvested 72 h later.

siRNA synthesis. One siRNA against *ANO5* was chemically synthesized and the sequences were listed as follows: siRNA-1269, 5'-GCU GUA GUU GGC UUA GCU UTT-3'; siRNA-2487, 5'-GCU CAU AGC AUA GGU GUU UTT-3'. The non-targeting nucleotides were used as a negative control siRNA-NC, 5'-UUC UCC GAA CGU GUC ACG UTT-3'.

In vitro migration and invasion assays. Cell migration/invasion assays were performed using 24-well Transwells (6.5 mm pore size, Costar), coated without (migration) or with (invasion) Matrigel. TPC-1 and FTC-133 cells were starved in serum-free media for 12 h, trypsinized and washed three times in RPMI-1640/DMEM containing 0.1% BSA. Cells ($1x10^4$) were seeded into the upper chamber, and 600 μl medium containing 10% FBS was placed in the lower chamber at 37˚C in 5% CO_2. After 48 h incubation, Matrigel and cells remaining in the upper chamber were removed. Cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with Coomassie Brilliant Blue, photographed and counted under a dissecting microscope. Every experiment was repeated three times.

Gene set enrichment analysis (GSEA). GSEA was carried out using GSEA software according to literature (21,22). Firstly we classified thyroid cancer samples (GSE3678) into *ANO5* high expression and *ANO5* low expression group according to *ANO5* expression, subsequently three gene sets including KEGG, Hallmark and BioCarta were chosen to conduct GSEA.

Statistical analysis. The statistical difference of quantitative variables was evaluated with Student's t-test using GraphPad Prism 5 software and R x64 3.2.2 software, $p<0.05$ was considered statistically significant.

Results

ANO5 is downregulated in thyroid cancer. In order to find differentially expressed genes in thyroid cancer and adjacent normal tissue, a GEO dataset (GSE3678) which contains seven cancerous and seven normal tissues was chosen. A total of 38 genes were discovered to display at least 3-fold alterations (Fig. 1A). Furthermore, we evaluated expression of these 38 genes in clinical thyroid cancer tissues and adjacent noncancerous tissues by real-time PCR (Fig. 1B). We found that the expression of some genes including *TPO* (23), *ANO5*, *ERBB4* (24) and *SLC4A4* (25) genes were in accordance with the results of GEO gene expression atlas (Fig. 1A), then we focused on *ANO5* and its family through biological information analysis and previous literature studies. In order to investigate the role of anoctamin family in thyroid cancer progression, we first measured the expression profile of this family in thyroid cancer tissue samples by mining a public database (GSE3678). Interestingly, only *ANO5* was significantly downregulated in thyroid cancer compared to adjacent noncancerous tissues (Fig. 1C). Subsequently we confirmed that 69.5% (57/82) thyroid cancer showed up to 2-fold downregulation of *ANO5* by real-time PCR assay (Fig. 1D). Similarly, western blot assay also proved that *ANO5* is downregulated in PTC and follicular thyroid cancer compared to adjacent noncancerous tissues (Fig. 1E). Collectively, these data revealed that *ANO5* expression is significantly downregulated in thyroid cancer.

Table I. Real-time PCR primers utilized in this study.

Genes	Sequences
ADH1B	F: CCCGGAGAGCAACTACTGC
	R: AACCAGTCGAGAATCCACAGC
ANO5	F: TTTTGGAAACAACGACAAGCCA
	R: ACCATACTGGTGACGACAAGAG
BMP2	F: ACCCGCTGTCTTCTAGCGT
	R: TTTCAGGCCGAACATGCTGAG
CDH16	F: GTCCCTAGAGCCTATCCACCT
	R: TGCATTCACTTCAAAGGGTCC
CLCNKB	F: GCCCTCCTTCTATGATGGCAC
	R: CCTGCCCTTGGTGACAGTG
DLG2	F: CCTCTACGTCAGAGCCATGTT
	R: ATCGGGCACGTTCCTTTCTTT
DPP6	F: CTACGCCGCCATCAATGATTC
	R: GGGATAGTGGTAGGGCTTCAC
DPY19L2	F: CTTCCAGTTCGTCCGTAATTCC
	R: TCTCCCGTTCCAAAGATGAGAG
EDN3	F: GGGACTGTGAAGAGACTGTGG
	R: AGACACACTCCTTGTCCTTGTA
ERBB4	F: GTCCAGCCCAGCGATTCTC
	R: AGAGCCACTAACACGTAGCCT
ESRRG	F: GCCCTCACTACACTGTGTGAC
	R: CCTGCTAATTTGGACTGGTCTT
FABP4	F: ACTGGGCCAGGAATTTGACG
	R: CTCGTGGAAGTGACGCCTT
FHL1	F: AAGAACCGCTTCTGGCATGAC
	R: CCCCTTGTACTCCACGTTTTG
FOS	F: CCGGGGATAGCCTCTCTTACT
	R: CCAGGTCCGTGCAGAAGTC
GHR	F: CCATTGCCCTCAACTGGACTT
	R: AATATCTGCATTGCGTGGTGC
GNA14	F: GAGCGATGGACACGCTAAGG
	R: TCCTGTCGTAACACTCCTGGA
GPM6A	F: ATTCCCTATGCCTCTCTGATTGC
	R: GCCATCTCAAAGTAGGTTTGCAG
HGD	F: ATTTACACCGAGTTTGGCAAGA
	R: GGTCTCCTCAAAGACATCTATGC
ITPR1	F: ATTGCTGGGGACCGTAATCC
	R: TCCAATGTGACTCTCATGGCA
KIAA1324	F: GGAGCTTCATGCCTGCAAAGA
	R: CATCAAACCGAATGCCTGTGC
LIFR	F: TGGAACGACAGGGGTTCAGT
	R: GAGTTGTGTTGTGGGTCACTAA
LRP2	F: GTTCAGATGACGCGGATGAAA
	R: TCACAGTCTTGATCTTGGTCACA
PID1	F: CGTGGAGTGCGAGAGCAAG
	R: CTGGGAAACCTCTTCGGAGGA
PLA2R1	F: TAAATCGGTTCTGACCCTGGA
	R: GCCACCGTAAGGAAACGAG
PTHLH	F: AAGGTGGAGACGTACAAAGAGC
	R: CAGAGCGAGTTCGCCGTTT

Table I. Continued.

Genes	Sequences
RYR2	F: CATCGAACACTCCTCTACGGA
	R: GGACACGCTAACTAAGATGAGGT
SLC26A4	F: TGGTGGGATCTGTTGTTCTGA
	R: GGATCTGCCAAGTACCTCACT
SLC26A7	F: GTGACCCAAGGATTGGCCTTT
	R: GGCAACATGATGTCCCATTCC
SLC4A4	F: GGGTGCCCTGACTGAAGTTC
	R: GGTCGTGCCTGTCTTTTGCT
TFF3	F: CCAAGCAAACAATCCAGAGCA
	R: GCTCAGGACTCGCTTCATGG
TMEM171	F: AACCGCTAAACGAGACAGACA
	R: ACACAATCCCACAAGCACAATC
TMPRSS3	F: TGGAAGGGTCACTACGCAAAT
	R: AGTGGTGTAATGCAGTCACCT
TNFRSF11B	F: GCGCTCGTGTTTCTGGACA
	R: AGTATAGACACTCGTCACTGGTG
TPO	F: GCCAACAAGCGGAGTGATTG
	R: GGGCAGCATGTAAGGGAGAC
TPPP	F: AGGGGTGACGAAAGCCATC
	R: CGGACACATAGCCTGACTCG
WSCD2	F: AAACCTGTGCGCTTCTTTACC
	R: GTACCTGCGAGCAATGCTTGA
KLK7	F: TAATGACCTCATGCTCGTGAAGC
	R: CAGCCGGAGACAGTACAGG
GAPDH	F: CTGGGCTACACTGAGCACC
	R: AAGTGGTCGTTGAGGGCAATG

F, forward; R, reverse.

Downregulation of ANO5 is positively associated with lymph node metastasis of thyroid cancer. In order to explore the correlationship between downregulation of *ANO5* and clinical characteristics, we analyzed RNA-seq data of thyroid cancer from TCGA database. We found that *ANO5* expression is significantly associated with lymph node stage (N0 or N1, p=0.01) and neoplasm histologic type (follicular or classical/papillary, p=6.97E-05) (Table II), *ANO5* expression levels in thyroid cancer with lymph node metastasis is lower than that without lymph node metastasis (Fig. 2A). Real-time PCR also confirmed the downregulation of *ANO5* in thyroid cancer with lymph node metastasis compared to that without lymph node metastasis (Fig. 2B). In addition, we proved, as shown in Table III, *ANO5* expression was significantly associated with lymph node metastasis (lymph node negative vs. lymph node positive, p=0.0038, χ^2=8.376) in our in-house samples. There are no significant association between *ANO5* expression and other tumor characteristics, such as age, gender, size, Hashimoto background or multifocal disease (Table III). In order to carry out cellular and functional experiments, we next evaluated *ANO5* expression in thyroid cancer cell lines, including FTC-133 and TPC-1, by real-time PCR, data show

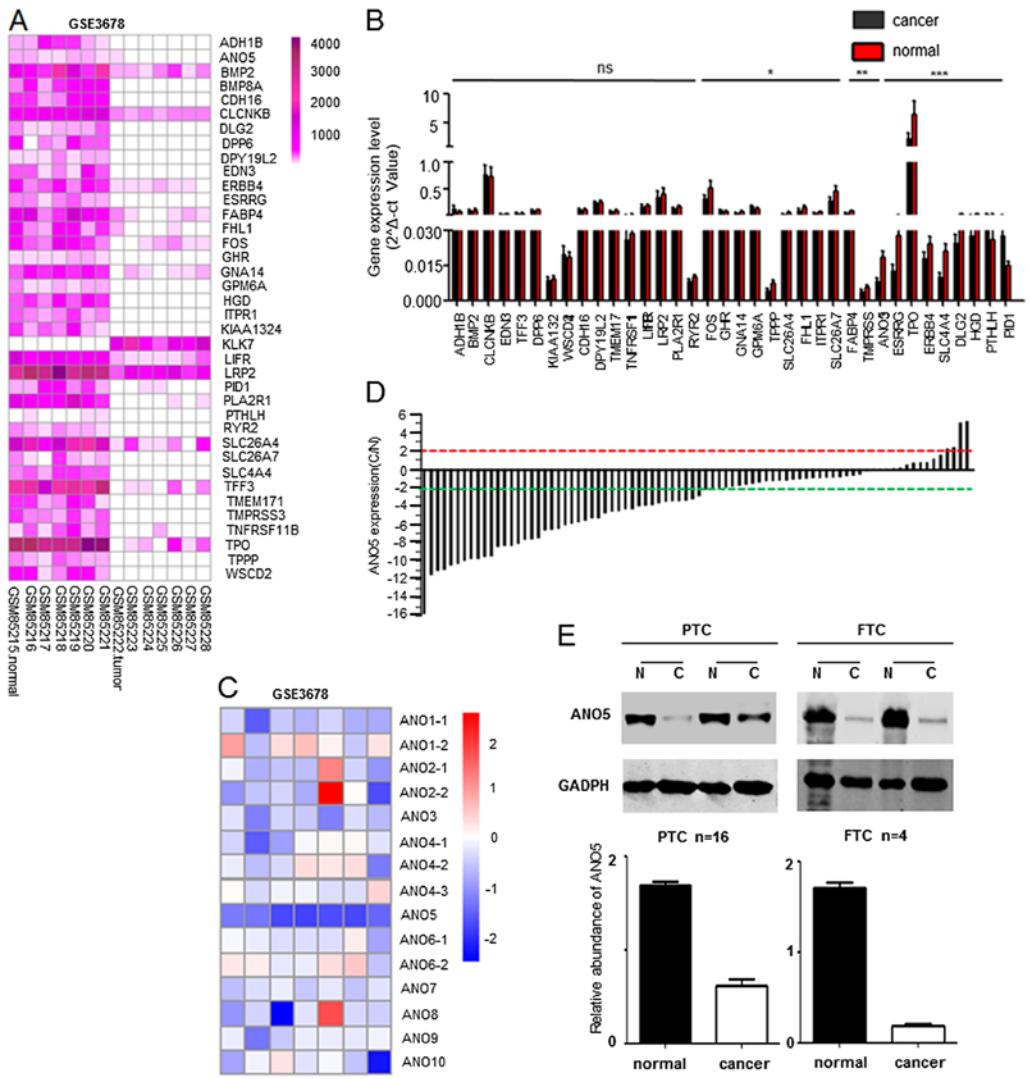

Figure 1. Anoctamin5 (*ANO5*) is downregulated in thyroid cancer. (A) Result of hierarchical clustering for top 38 differential expressed genes. (B) Real-time PCR assay validation of top 38 differential expressed genes in clinical thyroid cancer tissues. (C) The expression ratio of ANO family members in thyroid cancer and paired noncancerous tissue was calculated by mining public database (GSE3637). Negative value indicates gene expression is downregulated in thyroid cancer. (D) Real-time PCR assay was utilized to evaluate the expression of ANO5 in 82 paired thyroid cancer tissues. C, indicates thyroid cancer samples; N, represents paired noncancerous tissues. Green and red lines separately mean that gene expression is downregulated or upregulated twofold in thyroid cancer. (E) Western blotting was used to detect ANO5 expression in 16 paired papillary thyroid cancer (PTC) and 4 paired follicular thyroid cancer (FTC). *p<0.05. **p<0.01 and ***p<0.001. ns, no significant difference.

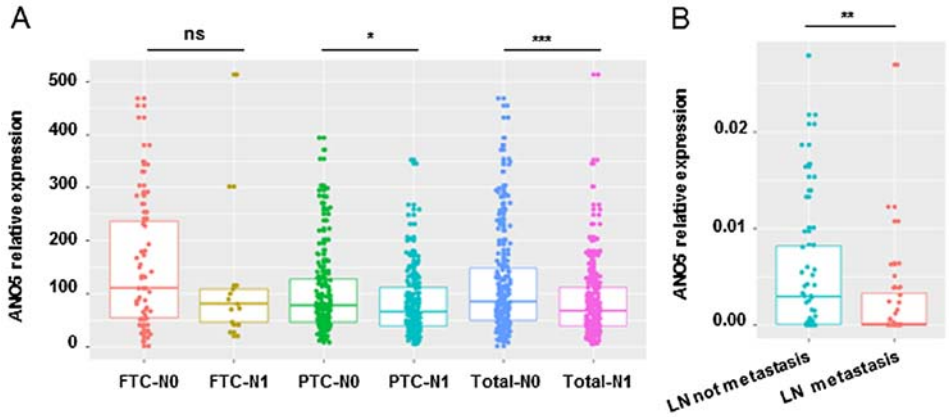

Figure 2. Downregulation of anoctamin5 (*ANO5*) is positively associated with lymph node metastasis. (A) TCGA data were used to compare *ANO5* expression in thyroid cancer with or without lymph node metastasis (56 samples FTC-N0, 12 samples FTC-N1, 133 samples papillary thyroid cancer (PTC)-N0 and 161 samples PTC-N1). N0 indicates no lymph node metastasis, N1 represents lymph node metastasis. (B) Real-time PCR assay was utilized to measure *ANO5* expression in 36 thyroid cancers with lymph node metastasis and 46 thyroid cancers without lymph node metastasis. *p<0.05. **p<0.01 and ***p<0.001. ns, no significant difference.

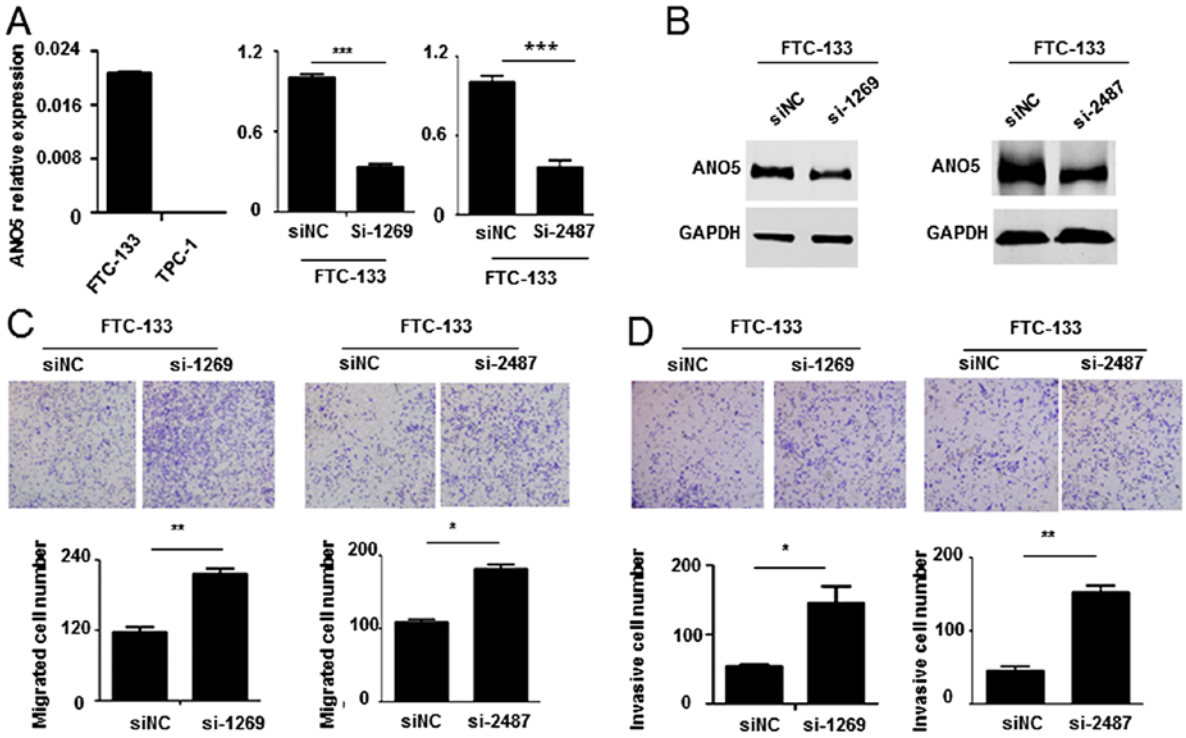

Figure 3. Knockdown of anoctamin5 (*ANO5*) promotes FTC-133 cell migration and invasion. (A) Real-time PCR was used to detect ANO5 expression in thyroid cancer cell lines. FTC-133 cells were transfected with siRNA against ANO5 (si*ANO5*), and siNC served as negative control. After 48 h, RNA was extracted and *ANO5* expression was evaluated with (A) real-time PCR and (B) western blotting. Transwell chambers coated without or with Matrigel were used to detect (C) migration or (D) invasion of FTC-133 cells (up). The number of cells was counted and compared (bottom). *p<0.05. **p<0.01 and ***p<0.001.

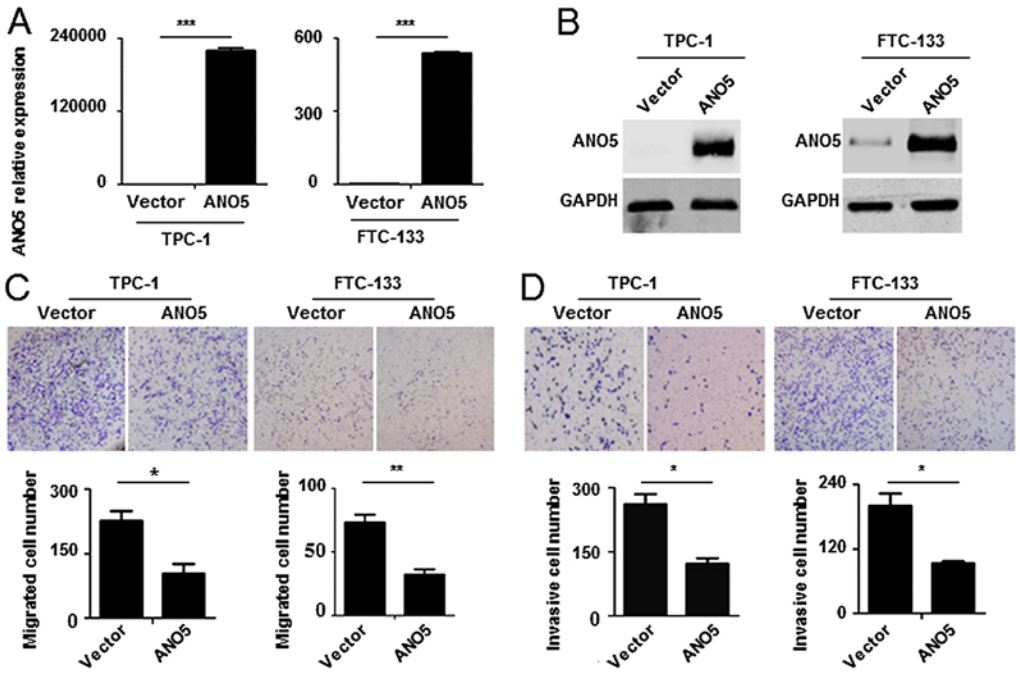

Figure 4. Overexpression of anoctamin5 (*ANO5*) inhibits FTC-133 and TPC-1 cells migration and invasion. TPC-1 and FTC-133 cells were infected with lentivirus expressing *ANO5*. After 72 h, RNA was collected, (A) real-time PCR and (B) western blot assays were used to measure *ANO5* expression. Empty vector (vector) served as negative control. (C) Transwell assay was used to test the effect of *ANO5* overexpression on FTC-133 and TPC-1 cell migration (up). Transferred cells were counted and compared (bottom). (D) Invasiveness of FTC-133 and TPC-1 cells was measured in Transwell chambers coated with Matrigel (up), and cells were counted and compared (bottom). *p<0.05. **p<0.01 and ***p<0.001.

that *ANO5* is undetectable in PTC cell line (Fig. 3A). In total, these findings indicate that downregulation of *ANO5* is positively associated with lymph node metastasis of thyroid cancer.

Knockdown of ANO5 promotes FTC-133 cell migration and invasion. To understand whether *ANO5* knockdown affects thyroid cancer cells migration and invasion, we

Table II. The correlation of ANO5 expression with clinical characteristics of thyroid cancer from TCGA.

Clinical characteristic	No. of patients	ANO5 High	ANO5 Low	p-value
Age (years)				
≥60	117	65	52	
<60	379	183	196	0.169
Gender				
Male	134	62	72	
Female	362	186	176	0.312
Recurrence				
Yes	46	22	24	
No	436	215	221	0.848
Overall survival (month)				
≥60	97	41	56	
<60	399	207	192	0.090
Neoplasm histologic type				
Classical/usual	354	161	193	
Follicular	100	68	32	6.97E-05
Tall cell	35	16	19	0.990
Tumor stage				
T1	141	71	70	
T2	164	86	78	0.717
T3	167	79	88	0.594
T4	22	10	12	0.669
Metastasis stage				
M0	276	136	140	
M1	9	5	4	0.711
Lymph node stage				
N0	224	122	102	
N1	222	94	128	0.010

Table III. The correlation of ANO5 expression with clinical characteristics of PTC.

Clinical characteristic	No. of patients	ANO5 expression (ct) >30	ANO5 expression (ct) ≤30	p-value	χ^2
Age (years)					
<45	32	17	15	0.3244	0.971
≥45	50	21	29		
Gender					
Male	25	14	11	0.2456	1.349
Female	57	26	33		
Microcarcinoma					
Yes	33	16	17	0.6846	0.165
No	49	26	23		
Hashimoto's thyroiditis					
Yes	43	20	23	0.8415	0.04
No	39	19	20		
Multifocal					
Yes	29	13	16	0.2356	1.407
No	53	31	22		
LN metastasis					
Yes	32	22	10	0.0038	8.376
No	50	18	32		

T indicates papillary thyroid cancer tissues. Ct=30 is the average of cancer tissues.

synthesized siRNA against *ANO5* (si*ANO5*) and transfected FTC-133 cells which have higher *ANO5* expression. Real-time PCR and western blot results indicate that siRNA significantly decreased *ANO5* expression (Fig. 3A and B). Cell migration assay showed that knockdown of *ANO5* increased the migrated cell number (Fig. 3C). Meanwhile, inhibition of *ANO5* also promoted FTC-133 cell invasion (Fig. 3D). Our results prove that knockdown of *ANO5* promotes FTC-133 cell migration and invasion.

Overexpression of ANO5 inhibits FTC-133 and TPC-1 cell migration and invasion. Next we detect the effect of *ANO5* overexpression on thyroid cancer cell migration and invasion. Lentivirus expressing *ANO5* was constructed and TPC-1 and FTC-133 cells infected. Real-time PCR and western blotting proved that *ANO5* successfully expressed in TPC-1 and FTC-133 cells (Fig. 4A and B). Cell migration and invasion assays showed that ectopic expression of *ANO5* decreased the invasive and migrated cell number of TPC-1 and FTC-133 cells (Fig. 4C and D). Collectively these data demonstrate that overexpression of *ANO5* inhibits FTC-133 and TPC-1 cell migration and invasion.

ANO5 activates the JAK/STAT3 pathway in thyroid cancer. To explore the mechanism by which *ANO5* regulates thyroid cancer cells migration and invasion, we carried out GSEA using public datasets (GSE3678). We found that lower *ANO5* expression was negatively associated with some important signaling pathways such as *TPO*, *KRAS*, *p53* and *VEGF* (Fig. 5A). In addition, the results also showed that lower *ANO5* expression was positively associated with *JAK/STAT3* pathway which is well-known to be activated during cancer metastasis (19,20) (Fig. 5B). Western blot results indicated that overexpression of *ANO5* suppressed phosphorylation of *STAT3* but silencing of *ANO5* increased the phosphorylation of *STAT3* (Fig. 5C). Collectively these data demonstrated that *ANO5* can regulate *JAK/STAT3* signaling pathway in thyroid cancer.

Discussion

Most of thyroid cancers are well differentiated and have good prognosis, but lymph node metastasis usually increase the risk of recurrence and mortality (3-5). In this study, for the first time, we identified that *ANO5* gene was documented to be expressed in 7 papillary thyroid carcinoma samples.

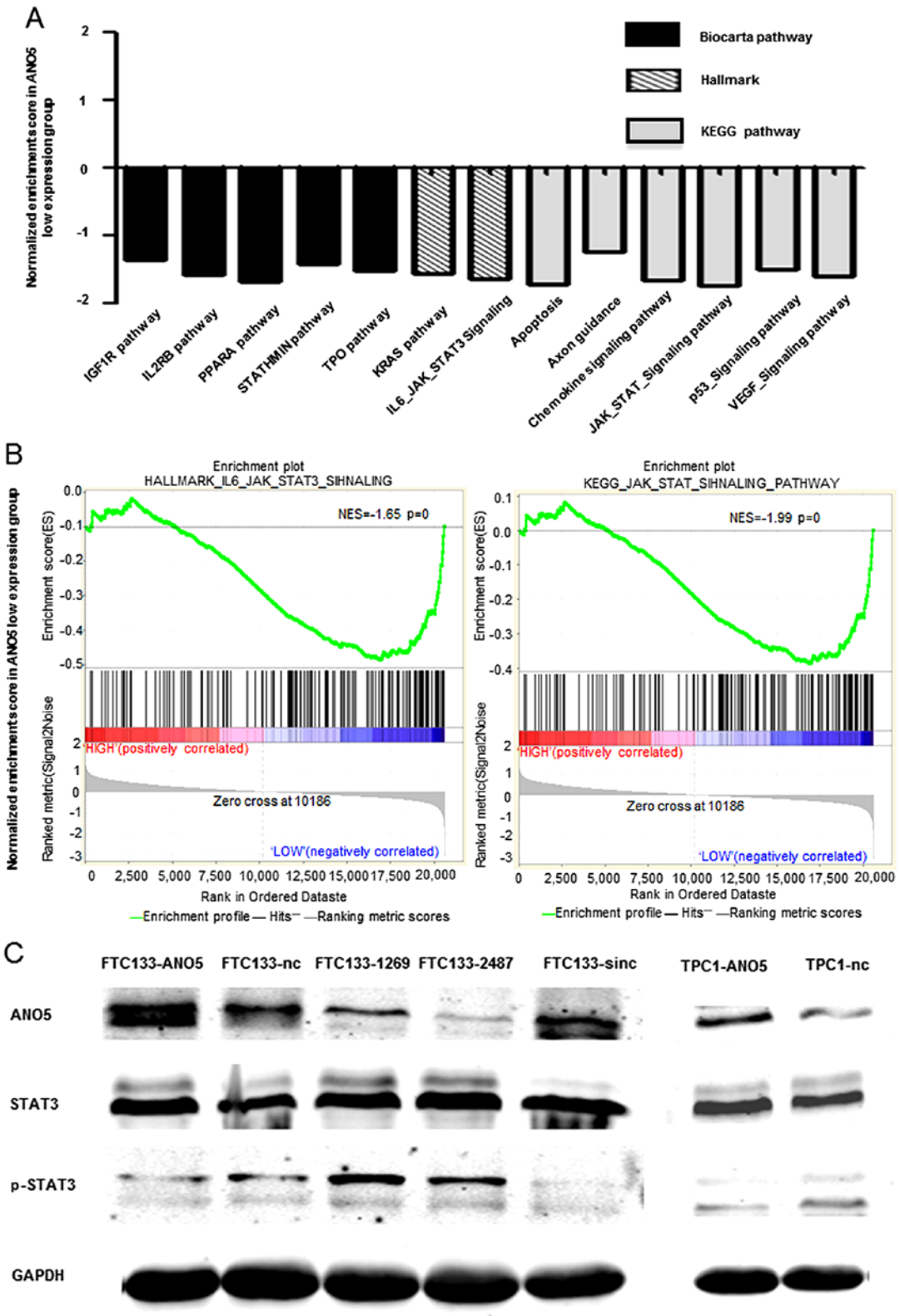

Figure 5. Anoctamin5 (*ANO5*) activates the *JAK/STAT3* pathway in thyroid cancer. The metastasis pathway was activated in thyroid cancer patients having lower *ANO5* expression. (A) Public dataset (GSE3678) was the lower *ANO5* expression group, and the normalized enrichment score of three gene set categories was calculated by gene set enrichment analysis (GSEA). Each column represented one statistically significant gene set ($p<0.05$). (B) GSEA was performed in thyroid cancer tissues with lower *ANO5* expression versus higher *ANO5* expression based on the gene sets of the *JAK/STAT3* pathway. Two gene sets including metastasis and negative regulation of metastasis were activated in thyroid cancer patients with lower ANO5 expression, NES represents normalized enrichment score. (C) Western blotting was used to detect the effect of *ANO5* on *JAK/STAT3* signaling pathway. Overexpression of *ANO5* can inhibit the phosphorylation of *STAT3*, while *ANO5* knockdown activates the phosphorylation of *STAT3*.

Moreover, TCGA databases showed that expression of *ANO5* in PTC with LN metastases (n=166) is lower than those without LN metastases (n=185) (Fig. 2B). We found *ANO5* is downregulated in thyroid cancer tissues including PTC and

FTC (Fig. 1E), downregulation of *ANO5* promotes thyroid cancer cell migration and invasion (Fig. 3C and D), while overexpression of *ANO5* has the opposite effect (Fig. 4C and D). Identifying the molecular events that regulate thyroid cancer metastasis holds promise for developing more effective prevention for human thyroid cancer. One of the major signaling pathways that is aberrantly activated and is critical for thyroid tumor metastasis is the *JAK/STAT3* pathway by GSEA (Fig. 5B). The phosphorylated STAT3 protein can translocate into the nucleus, where it activates the transcription of various genes that regulate vital cellular functions, including cell proliferation and metastasis (26). These data suggested a relationship between *ANO5* and *JAK/STAT3* pathway activation, but it remained to be determined if the *JAK/STAT3* pathway is required for thyroid cancer metastasis. These data indicated that *ANO5* is a potential tumor suppressor gene in thyroid cancer, and downregulation of *ANO5* participates in lymph node metastasis. Thus, the functional effect of *ANO5* in PTC metastasis and has potential clinical value for developing gene therapy to treat PTC and subsequent lymph node or distant metastases and improving prognosis.

Other than *ANO5*, some *ANO* family members such as *ANO1-4* and *ANO6-10* have been reported to be related to tumors. Previous studies reported that *ANO1*, another member of anoctamin family, is upregulated in gastrointestinal stromal tumors (27) and head and neck squamous cell carcinomas (28), inhibition of *ANO1* can suppresses tumor invasion (29-32). We measured *ANO5* expression in PTCs using a public database, and found that in contrast to *ANO1* expression, *ANO5* is downregulated in cancer tissues (Fig. 1C). Additional study of *ANO5* revealed that it negatively regulate lymph node metastases of PTC, a role opposing that of *ANO1* in other tumors (33,34). It has been reported that overexpression of *ANO6* also increase cancer cell migration (12). ANO7 has been reported to participate in the development of breast (35) and prostate (36) cancers, the monoantiboby targeting the extracellular regions of ANO7 has a potential application for immunotherapy (37). *ANO5* itself functions as a Cl^- channel, but its activation requires higher Ca^{2+} concentration than other ANO members (38). Thus, some antagonism may exist among different ANO family members with respect to tumors, and this warrants further study.

In conclusion, we identified that *ANO5* is downregulated in thyroid cancer and downregulation of *ANO5* promotes thyroid cancer cell migration and invasion. In addition, we found that the expression level of *ANO5* was correlated with activation of *JAK/STAT3* pathway in thyroid cancer, suggesting a potential application of *ANO5* as a biomarker. Altogether, our results demonstrate that targeting JAK/STAT3 pathway, using siRNA knockdown of *ANO5*, effectively promote lymph node metastasis of thyroid cancer, therefore, could be a potential novel therapeutic approach for treating lymph thyroid cancer. Thus, we confirmed that *ANO5* is a novel potential biomarker of thyroid cancer and its expression correlates with lymph node metastasis. To further uncover the effect of *ANO5* on proliferation and cell cycle and the detailed molecular mechanism of lymph node metastasis of thyroid cancer is necessary for clinical gene therapy in the future.

Acknowledgements

We would like to thank Lei Ye (Rui Jin Hospital) for providing us with TPC-1 and FTC-133 cells. This study was supported by the grants from the National Natural Science Foundation of China (grant nos. 81472501 and 81502197).

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