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Silencing of AHNAK2 restricts thyroid carcinoma progression by inhibiting the Wnt/β-catenin pathway

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AHNAK nucleoprotein 2 (AHNAK2) has been proposed to have an oncogenic role in various human cancers. However, the functional role of AHNAK2 in thyroid carcinoma (TC) progression has never been explored. In this study, quantitative real-time polymerase chain reaction and western blot were conducted to evaluate the expression of genes. The functional role of AHNAK2 was elucidated by cell count kit-8, colony-forming assay, wound-healing assay, and Transwell invasion assay. We found that AHNAK2 was highly expressed in thyroid carcinoma, and it was tightly correlated with the pathological stage in TC. The mRNA and protein levels of AHNAK2 were increased in TC cells. Silencing of AHNAK2 restricted the proliferation, metastasis, and epithelial-mesenchymal transition (EMT) of TC cells. AHNAK2 silencing inhibited the protein expression of β-catenin and cyclin D1, and AHNAK2 overexpression had the opposite effects. Moreover, LiCl or ICG-001 exposure counteracted the effects of AHNAK2 silencing or upregulation on malignant phenotypes of TC cells. In conclusion, the knockdown of AHNAK2 restrained the proliferation, metastasis, and EMT of TC cells by inhibiting the Wnt/β-catenin pathway, providing a new potential mechanism of AHNAK2 in understanding the oncogenesis and progression of TC.

Key words: AHNAK nucleoprotein 2, thyroid carcinoma, epithelial-mesenchymal transition, Wnt/β-catenin pathway

Thyroid cancer (TC) is one of the common malignancies occurring in the endocrine system, and its incidence continues to rise in the past 40 years [1]. Papillary thyroid carcinoma is the most common histological type, occupying about 90% of TC [2]. Although most TC patients can be treated by surgical operation and radioactive iodine therapy, some patients were refractory to radioactive iodine therapy and were easy to metastasize, which results in a higher mortality rate [3]. With the rapid development of molecular biology and genetic engineering, gene therapy has become a novel therapeutic strategy for TC treatment [4–6]. Thus, a detailed understanding of the molecular mechanism that drives TC aggravation has considerable significance for seeking a novel therapeutic target for TC.

AHNAK nucleoprotein 2 (AHNAK2) is a cytoskeletal protein that locates on the chromosome 14p32 and is expressed in a wide range of cell types. AHNAK2 is a member of the AHNAKs family that participates in the regulation of cytoarchitecture and calcium signaling [7]. AHNAK2 expression is upregulated in pancreatic ductal adenocarcinoma (PDAC) tissues and is correlated with the overall survival rate of patients with PDAC [8]. Recently, AHNAK2 was reported to be aberrantly expressed in various human cancers, indicating that AHNAK2 might play a crucial role in the progression of tumorigenesis [9, 10]. As an example, Wang and his colleagues found that AHNAK2 was highly expressed in clear cell renal cell carcinoma and its upregulation was associated with tumor development and patients' poor prognosis. Moreover, they also suggested that upregulation of AHNAK2 induced by hypoxia promoted the progression of clear cell renal cell carcinoma by inducing epithelial-mesenchymal transition (EMT) and stem cell-like properties [11]. Additionally, Li *et al.* have indicated that the upregulation of AHNAK2 was tightly correlated with the overall survival time of patients with uveal melanoma, and downregulation of AHNAK2 impaired the proliferation, migration, and invasion of M17 and SP6.5 cells by inducing the inactivation of phosphatidylinositol 3-kinase signaling [12]. Furthermore, Kim *et al.* suggested that TC patients with a higher expression of AHNAK2 displayed a poor recurrence-free survival rate compared with the patients with a lower expression of AHNAK2, revealing that AHNAK2

serves as a major player in TC development [13]. However, although the oncogenic role of AHNAK2 has been elucidated in several types of human cancers, its functional role in TC progression remains elusive.

In the present study, we sought to elaborate the functional role of AHNAK2 in TC progression and found that silencing of AHANK2 restricted the proliferation and metastasis of TC cells. Mechanically, AHNAK2 presented its oncogenic role by inducing the activation of the Wnt/β-catenin signaling, unveiling a novel therapeutic target for TC.

Materials and methods

Cell culture and treatment. Human TC cell lines FTC-133 (Procell, Wuhan, China), BCPAP (Procell), TPC-1 (Procell), and human thyroid follicular epithelial cells (Nthyori3-1; Procell) were grown in RPMI-1640 medium plus 10% fetal bovine serum (Solarbio, Beijing, China), 100 U/ml penicillin from Solarbio, and 100 μg/ml streptomycin from Solarbio. These cells were kept at 37 °C in an incubator with 5% CO_2.

The full sequence of AHNAK2 was amplified and inserted into the pcDNA-3.2 vector and named the recombinant plasmid as AHNAK2-OE. si-AHNAK2 and si-NC were purchased from Santa Cruz (California, USA). Cell transfection was conducted using Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA) following the specifications of the manufacturer.

TPC-1 cells were transfected with si-AHNAK2 or si-NC for 48 h, followed by incubation with 20 mM of LiCl for 24 h. FTC-133 cells were transfected with AHNAK2-OE or OE-NC for 48 h, followed by incubation with 10 μM of ICG-001 for 24 h.

Quantitative real-time polymerase chain reaction (qRT-PCR). TPC-1 and FTC-133 cells were collected and assayed for RNA isolation using TRIzol reagent from Invitrogen, according to the manual of the kit. Afterward, cDNA synthesis was conducted using PrimeScript RT reagent Kit from Promega (Madison, WI, USA), following the product manual. The relative expression of AHNAK2 was determined with SYBR Green PCR Kit from Takara (Dalian, China), using GAPDH as the internal standard gene. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression of AHNAK2. The primers for the qRT-PCR assay are listed as follows: AHNAK2 (F: 5'-CGCGATGTGCGACTGC-3'and R: 5'-TGGTCATCTTCCGTTTCTGC-3') and GAPDH (F: 5'-GGAGCGAGATCCCTCCAAAAT-3'and R: 5'-GGCTGTTGTCATACTTCTCATGG-3').

Western blot. Proteins were isolated using RIPA buffer from Invitrogen and then quantified using a BCA kit (Beyotime, Beijing, China). Following this, the proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. Following incubation with 5% skim milk, the membrane was probed and shaken overnight at 4 °C with the primary antibodies against AHNAK2 (catalog number: ab70053), E-cadherin (catalog number: ab238099), N-cadherin (catalog number: ab18203), β-catenin (catalog number: ab223075), cyclin D1 (catalog number: ab226977), or GAPDH (catalog number: ab8245), followed by immunoblot with HRP-conjugated secondary antibodies (catalog number: goat anti-rabbit antibody ab7090, goat anti-mouse antibody ab47827). The immunoblots were developed by an enhanced chemiluminescence substrate from Beyotime and quantified using ImageJ software. All the antibodies used in this work were purchased from Abcam (Cambridge, UK).

Cell count kit-8 (CCK-8) assay. The viability of TPC-1 and FTC-133 cells was assessed by the use of a CCK-8 assay. In brief, TPC-1 and FTC-133 cells in the logarithmic phase were transfected with si-AHNAK2, AHNAK2-OE, or the corresponding controls, followed by the incubation with LiCl or ICG-001 for 24 h. Following this, TPC-1 and FTC-133 were incubated with CCK-8 solution from Solarbio and kept at 37 °C for 4 h. The absorbance of each well at 450 nm was measured using a microplate reader.

Colony-forming assay. After treatment, TPC-1 and FTC-133 cells were collected and dispersed with 0.25% trypsin. The cells suspended in RPMI-1640 medium containing 10% FBS were seeded onto a culture dish and then cultured at 37 °C in an incubator with 5% CO_2. Afterward, the cells were incubated for 2 weeks to form the visible colonies. After washing with PBS twice, the colonies were fixed for 15 min with methanol and then stained with Giemsa solution for 15 min. The number of colonies (<10 cells/colony) was manually counted employing a microscope.

Wound-healing assay. After treatment, TPC-1 and FTC-133 cells were plated onto 6-well plates, respectively. Afterward, a straight wound was introduced with a sterile 10 μl pipette tip, and PBS solution was used to remove cell debris. Subsequently, RPMI-1640 medium plus 10% FBS was added into each well and the plates were maintained at 37 °C in an incubator with 5% CO_2. The width of the scratch was measured at 0 h and 48 h after cell culture using ImageJ software.

Transwell invasion assay. After treatment, TPC-1 and FTC-133 cells were collected, dispersed, centrifuged, and suspended in a serum-free medium, followed by inoculation on the upper compartment of the Transwell chamber covered with Matrigel. The lower compartment was filled with the RPMI-1640 medium containing 10% FBS to induce cell invasion. After 24 h of incubation, the cells on the upper compartment were removed with a cotton swab, and the cells on the lower compartment were fixed with 4% paraformaldehyde and dyed with 0.1% crystal violet. Finally, the cells were counted employing an inverted fluorescence microscope.

Statistical analysis. Our data were represented as mean ± standard deviation and statistically analyzed with a one-way analysis of variance from GraphPad Prism 8 (GraphPad Software, USA). A probability value of $p<0.05$ was designated as the limit of statistical significance.

Results

AHNAK2 is upregulated in TC. As analyzed by the GEPIA database, we found that the expression of AHNAK2 was markedly increased in TC tissues compared with that in normal tissues (Figure 1A). Moreover, a striking correlation was discovered between AHNAK2 expression and pathological stage of TC (Figure 1B). Consistently, AHNAK2 was strikingly upregulated in TC cell lines relative to Nthy-ori3-1 cells, as determined by qRT-PCR assay (Figure 1C). Similarly, the upregulation of AHNAK2 in TC cells was confirmed by western blot (Figure 1D).

Silencing of AHNAK2 restricts the proliferation of TC cells. Since the highest expression of AHNAK2 was found in TPC-1 cells, and the lowest expression of AHNAK2 was observed in FTC-133 cells, loss-of-function and gain-of-function experiments were performed in TPC-1 and FTC-133 cells, respectively. We upregulated the expression of AHNAK2 in FTC-133 cells and knocked down the expression of AHNAK2 in TPC-1 cells to study the functional role of AHNAK2 in TC. As detected by western blot, the expression of AHNAK2 was substantially lower in TPC-1 cells transfected with si-AHNAK2 than that in the cells transfected with si-NC (Figure 2A). Also, an obvious elevation of AHNAK2 protein levels was noticed in FTC-133 cells transfected with AHNAK2-OE relative to the cells transfected with OE-NC (Figure 2B). Knockdown of AHNAK2 dramatically hampered the viability of TPC-1 cells, while forced expression of AHNAK2 markedly increased the viability of FTC-133 cells (Figures 2C, 2D). In line with this, the results of the colony-forming assay unveiled that silencing of AHNAK2 restricted the colony formation ability of TPC-1 cells, and upregulation of AHNAK2 enhanced the colony formation ability of FTC-133 cells (Figures 2E, 2F).

Silencing of AHNAK2 restricts the metastasis and EMT of TC cells. As determined by the wound-healing assay,

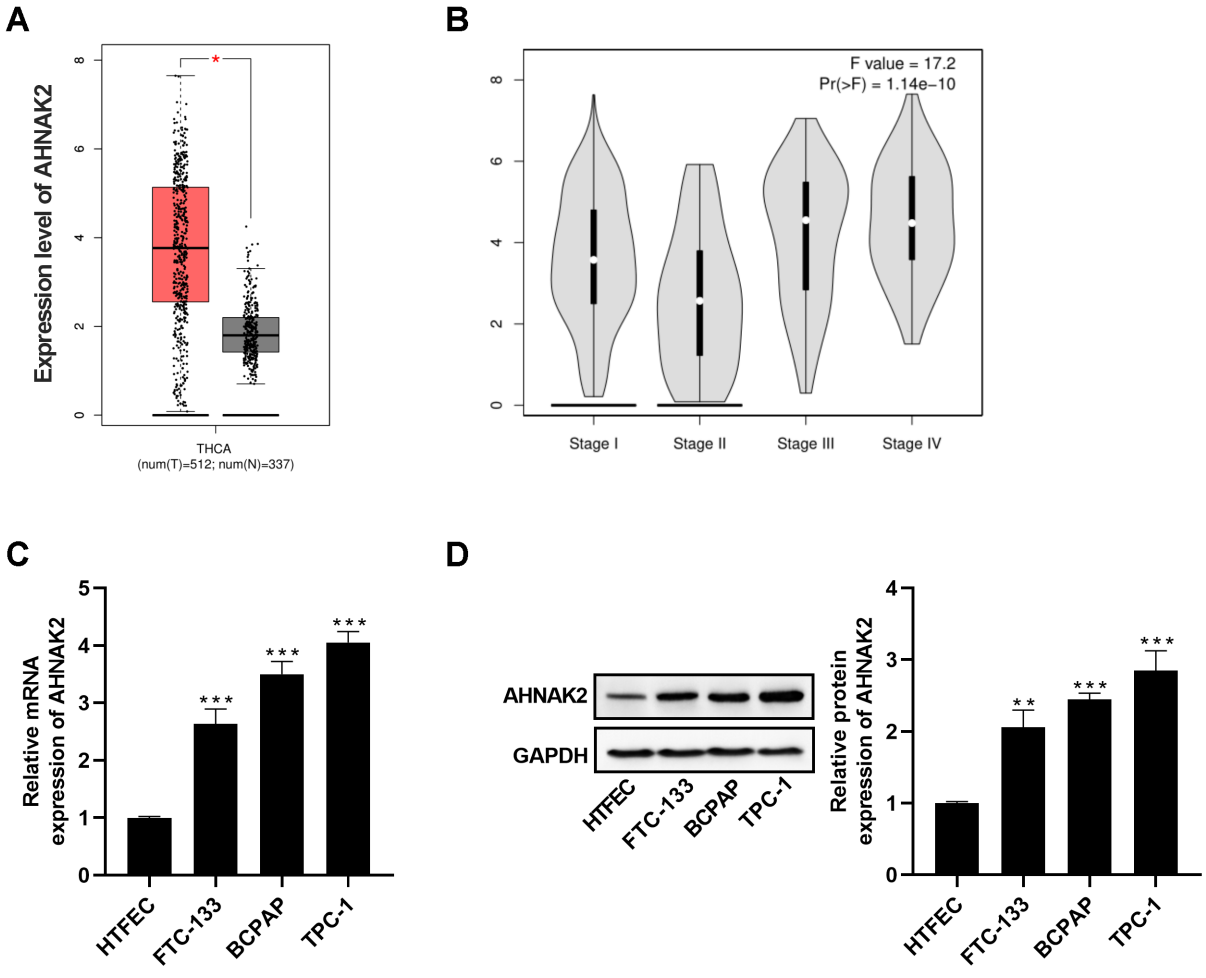

Figure 1. AHNAK2 is upregulated in TC. A) The GEPIA database analysis of the expression of AHNAK2 in TC tissues. B) The GEPIA database analysis of the correlation between AHNAK2 expression and pathological stage in TC. qRT-PCR (C) and (D) western blot analysis of the expression of AHNAK2 in TC cell lines. *p<0.05, ***p<0.001

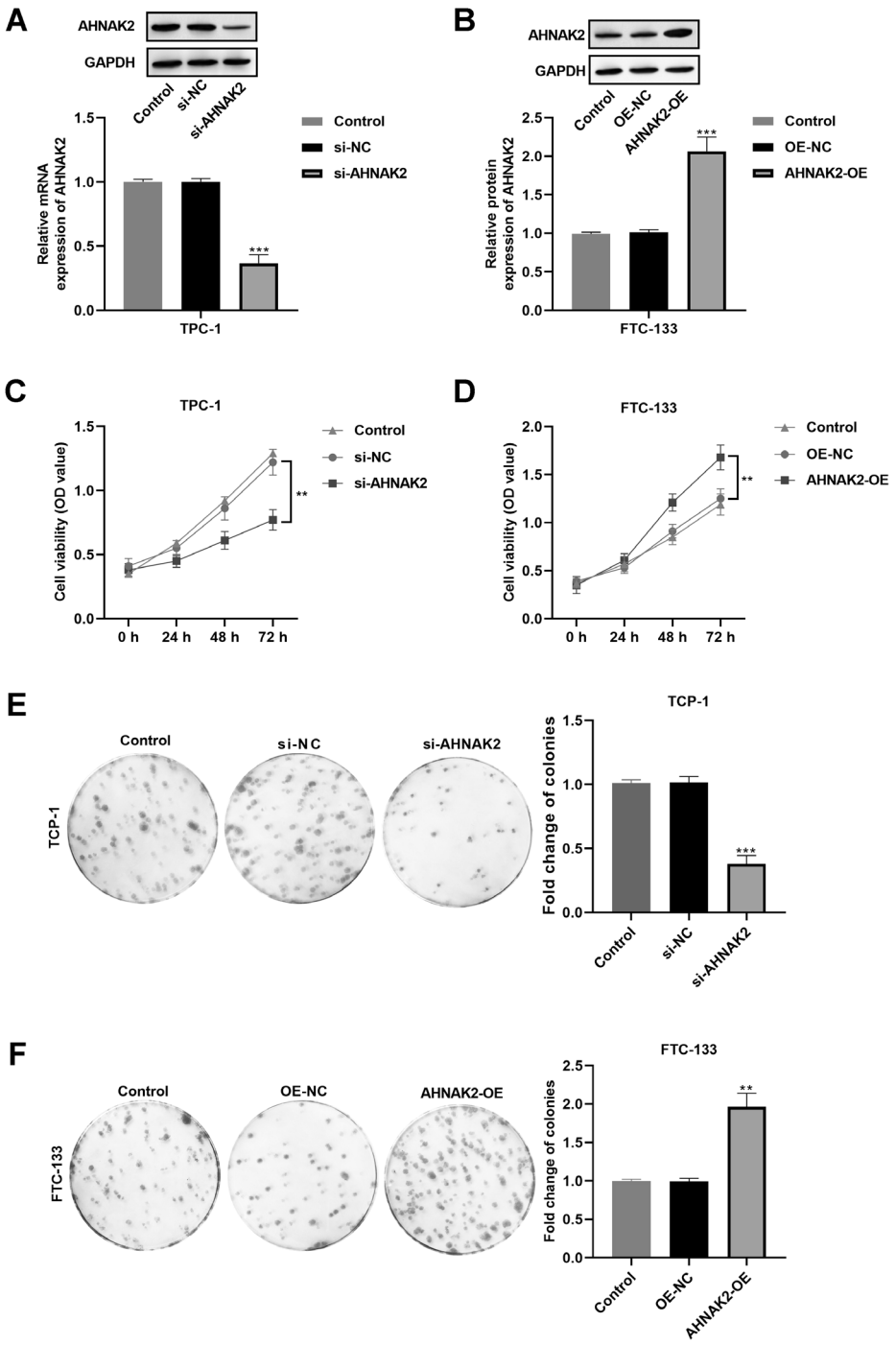

Figure 2. Silencing of AHNAK2 restricts the proliferation of TC cells. A) Western blot was conducted to confirm the silencing efficiencies of si-AHNAK2 in TPC-1 cells. B) Western blot was carried out to confirm the overexpression efficiencies of AHNAK2-OE in FTC-133 cells. C, D) CCK-8 assay was carried out to detect the viability of TPC-1 and FTC-133 cells. E, F) The proliferation of TPC-1 and FTC-133 cells was assessed by colony-forming assay. **p<0.01, ***p<0.001

deletion of AHNAK2 restrained the migration of TPC-1 cells, and upregulation of AHNAK2 promoted the migration of FTC-133 cells (Figures 3A, 3B). In parallel, the invasion of TPC-1 cells was restricted following si-AHNAK2 transfection, while the invasion of FTC-133 cells was enhanced after upregulation of AHNAK2 (Figures 3C, 3D). Similarly, we found that silencing of AHNAK2 increased the expression of E-cadherin, but decreased the expression of N-cadherin

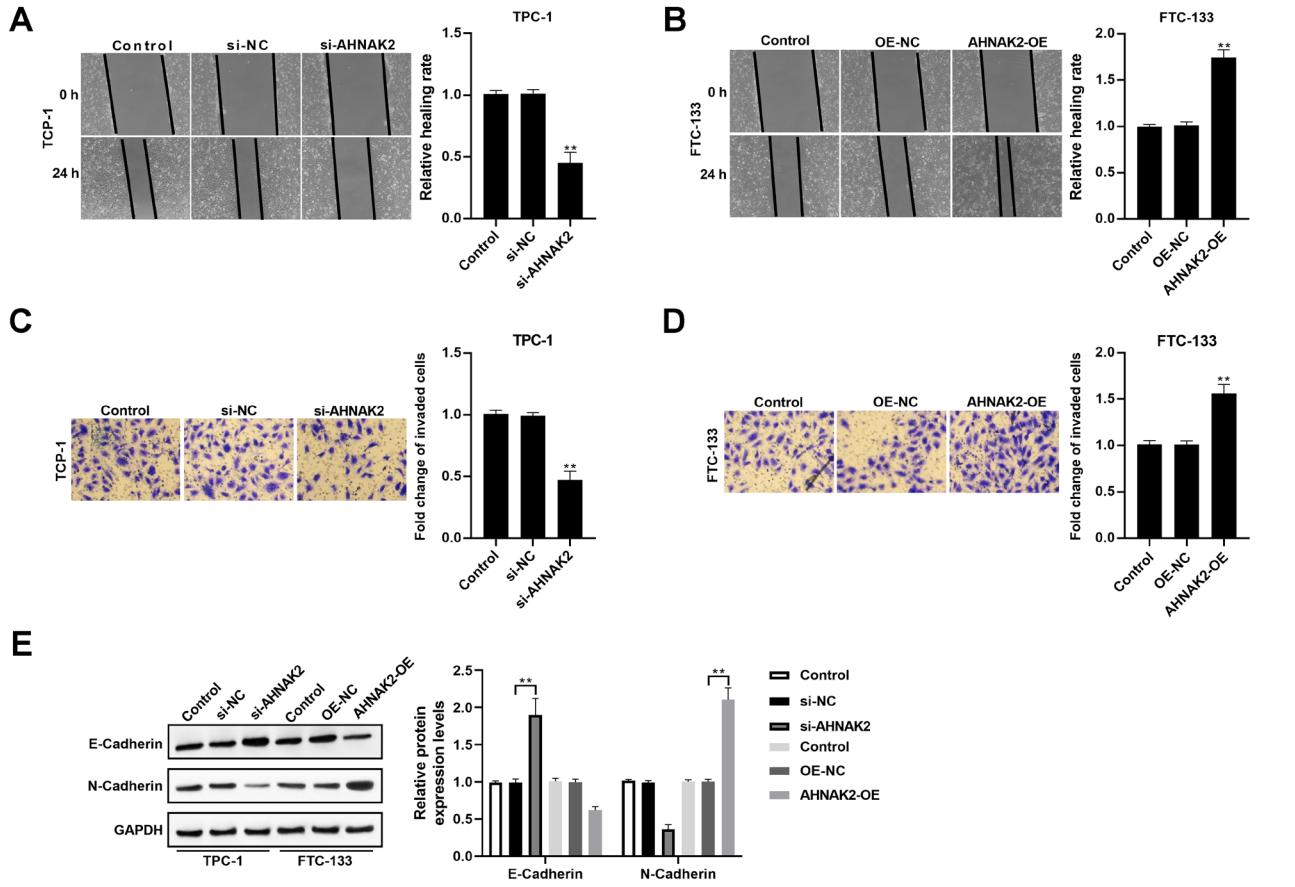

Figure 3. Silencing of AHNAK2 restricts the metastasis and EMT of TC cells. si-AHNAK2 and si-NC were transfected into TPC-1 cells, while AHNAK2-OE and OE-NC were transfected into FTC-133 cells. A, B) The migration of TPC-1 and FTC-133 cells was evaluated by the wound-healing assay. C, D) Transwell invasion assay was undertaken to measure the invasion of TPC-1 and FTC-133 cells. E) The expression of E-cadherin and N-cadherin was detected by western blot. **p<0.01

in TPC-1 cells. Meanwhile, overexpression of AHNAK2 reduced the expression of E-cadherin but elevated the expression of N-cadherin in FTC-133 cells (Figure 3E).

AHNAK2 participates in the activation of the Wnt/β-catenin pathway. To confirm if the Wnt/β-catenin pathway takes part in AHNAK2-mediated TC tumorigenesis, we evaluated the expression of β-catenin and Cyclin D1 by western blot. As a result, silencing of AHNAK2 memorably reduced the expression of β-catenin and Cyclin D1 in TPC-1 cells compared with that in TPC-1 cells stimulated with si-NC (Figure 4A). Conversely, compared with the OE-NC group, upregulation of AHNAK2 dramatically elevated the expression of β-catenin and Cyclin D1 in FTC-133 cells (Figure 4B).

AHNAK2 silencing represses the proliferation of TC cells via the Wnt/β-catenin pathway. In an attempt to determine if AHNAK2 silencing represses the proliferation of TC cells via the Wnt/β-catenin signaling pathway, we treated TPC-1 cells with LiCl at 48 h after si-AHNAK2 transfection and exposed FTC-133 cells to ICG-001 at 48 h after AHNAK2-OE transfection. As a result, activation of the Wnt/β-catenin signaling by LiCl could counteract the si-AHNAK2-induced reduction of TPC-1 cell viability, as indicated by the CCK-8 assay (Figure 5A). Inhibition of the Wnt/β-catenin signaling by ICG-001 blocked the elevation of FTC-133 cell viability induced by AHNAK2-OE (Figure 5B). Simultaneously, LiCl administration attenuated AHNAK2 silencing-induced reduction of the colony-formation ability of TPC-1 cells (Figure 5C). While, upregulation of AHNAK2 reduced the colony-formation ability of FTC-133 cells, and this reduction was mitigated by ICG-001 management (Figure 5D).

AHNAK2 silencing represses the metastasis and EMT of TC cells via the Wnt/β-catenin pathway. To further verify whether AHNAK2 silencing represses the metastasis and EMT of TC cells via the Wnt/β-catenin pathway, TPC-1 cells were treated with LiCl at 48 h after si-AHNAK2 transfection, and FTC-133 cells were exposed to ICG-001 at 48 h after AHNAK2-OE transfection. The results of the wound-healing assay unveiled that the deletion of AHNAK2 restrained the

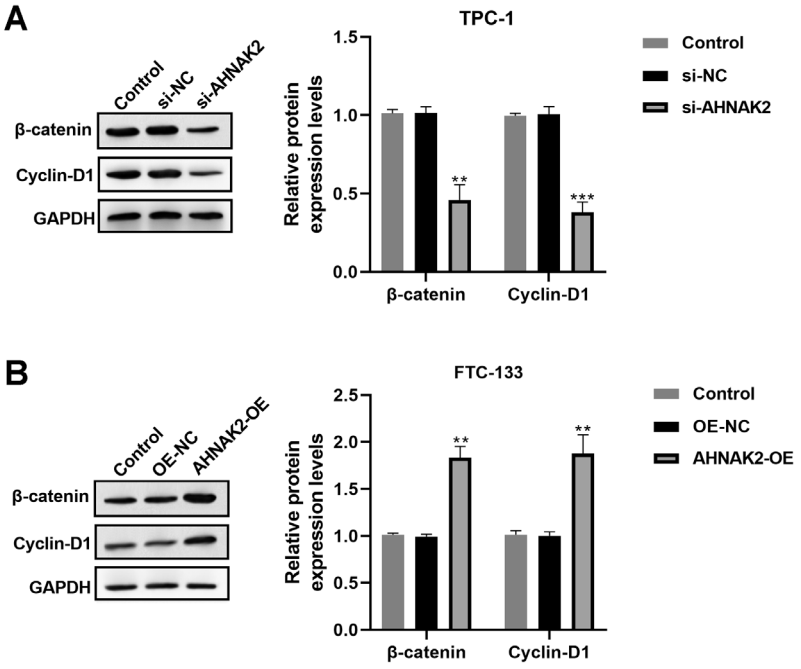

Figure 4. AHNAK2 participates in the activation of the Wnt/β-catenin pathway. si-AHNAK2 and si-NC were transfected into TPC-1 cells, while AHNAK2-OE and OE-NC were transfected into FTC-133 cells. A) Western blot analysis of β-catenin and Cyclin D1 expression in TPC-1 cells. B) Western blot analysis of β-catenin and Cyclin D1 expression in FTC-133 cells. **p<0.01

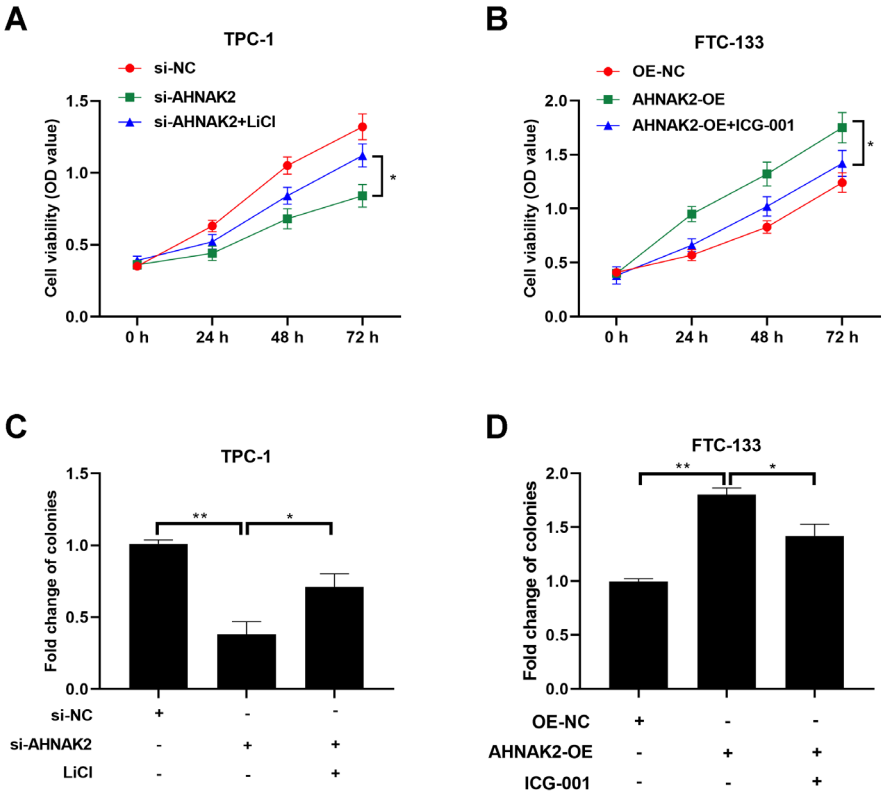

Figure 5. AHNAK2 silencing represses the proliferation of TC cells via the Wnt/β-catenin pathway. TPC-1 cells were transfected with si-AHNAK2 or si-NC, followed by incubation with LiCl. While FTC-133 cells were transfected with AHNAK2-OE or OE-NC, followed by incubation with ICG-001. A, B) CCK-8 assay was carried out to detect the viability of TPC-1 and FTC-133 cells. C, D) The proliferation of TPC-1 and FTC-133 cells was assessed by colony-forming assay. *p<0.05, **p<0.01

migration of TPC-1 cells, which was blocked by LiCl administration (Figure 6A). Upregulation of AHNAK2 promoted the migration of FTC-133 cells and this action was mitigated by ICG-001 treatment (Figure 6B). In parallel, AHNAK2 silencing-induced reduction of TPC-1 cell invasion was abrogated following LiCl treatment (Figure 6C), while administration of ICG-001 abolished AHNAK2 overexpression-induced elevation of cell invasion in FTC-133 cells (Figure 6D). Similarly, LiCl exposure counteracted AHNAK2 silencing-induced elevation of E-cadherin expression and reduction of N-cadherin expression in TPC-1 cells (Figure 6E). Also, upregulation of AHNAK2 reduced the expression of E-cadherin but elevated the expression of N-cadherin in FTC-133 cells, and these changes induced by AHNAK2 overexpression were conversed following ICG-001 administration (Figure 6E).

Discussion

Up to now, some patients with TC refractory to radioactive iodine therapy still lack effective treatment [14]. Hence, understanding the mechanism of TC and seeking a new therapeutic target is still necessary. AHNAK2 has been documented to be a momentous factor for the fibroblast growth factor 1 secretion signaling pathway, which serves as an important driver of tumorigenesis [15]. Notable, AHNAK2 was reported to be upregulated in TC and was correlated with advanced grades, stages, lymph node events as well as patients' survival rate, showing that AHNAK2 seems to be an independent prognostic factor in TC [16]. In our study, we found that AHNAK2 was upregulated in TC cells. Moreover, its upregulation was tightly correlated with the pathological stage in TC.

In the last few years, AHNAK2 was recognized as a new candidate tumor-promoting gene in a variety of human tumor types. For instance, the upregulation of AHNAK2 was discovered in lung adenocarcinoma and its expression was correlated with the poor prognosis of patients with lung adenocarcinoma, revealing that AHNAK2 might be a biomarker in lung adenocarcinoma [17, 18]. Consistently, Wang *et al.* have stated that silencing of AHNAK2 could restrain the proliferation and metastasis, and facilitated the apoptosis of lung adenocarcinoma cells A549 by inhibiting the activation of MAPK signaling pathway, showing the carcinogenic role of AHNAK2 in lung cancer [19]. Besides, knockdown of AHNAK2 hampered the migration, invasion, and EMT of lung adenocarcinoma cells by suppressing the transforming growth factor beta-induced smad3 signaling

Figure 6. AHNAK2 silencing represses the metastasis and EMT of TC cells via the Wnt/β-catenin pathway. TPC-1 cells were transfected with si-AHNAK2 or si-NC, followed by incubation with LiCl. While FTC-133 cells were transfected with AHNAK2-OE or OE-NC, followed by incubation with ICG-001. A, B) The migration of TPC-1 and FTC-133 cells was evaluated by wound healing assay. C, D) Transwell invasion assay was undertaken to measure the invasion of TPC-1 and FTC-133 cells. E) The expression of E-Cadherin and N-Cadherin was detected by western blot. *p<0.05, **p<0.01

pathway [20]. In gastric cancer, silencing of AHNAK2 has been documented to be closely linked with the hypersensitivity of the gastric cancer cells to chemotherapy [21]. Furthermore, Yang and his colleagues, using the integrated microarray analysis, have demonstrated that high expression of AHNAK2 was strikingly associated with the prognosis of patients with pancreatic cancer, indicating that AHNAK2 maybe acts as a promising prognostic biomarker for pancreatic cancer [22]. However, the excise role of AHNAK2 in TC has not yet been investigated. Given that TPC-1 cells showed a higher expression of AHNAK2 than FTC-133 cells, herein, we explored the functional role of AHNAK2 by silencing AHNAK2 in TPC-1 cells and overexpressing AHNAK2 in FTC-133 cells. We found that silencing of AHNAK2 restricted the proliferation, metastasis, and EMT of TPC-1 cells, while upregulation of AHNAK2 promoted the proliferation, metastasis, and EMT of FTC-133 cells, manifesting the tumor-promoting role of AHNAK2 in TC.

Wnt/β-catenin signaling has been highlighted as key signaling that plays a vital role in the regulation of cell processes, such as cell proliferation, differentiation, and migration [23]. It is well established that the Wnt/β-catenin signaling was implicated in a variety of human cancers. There is evidence to suggest that the activation of the Wnt/β-catenin signaling is closely related to the progression of TC. β-catenin has been identified as the downstream effector of the Wnt signaling, which can activate cyclin D1 to promote the growth and metastasis of TC [25]. Activation of the Wnt/β-catenin signaling could overturn the inhibitory effect of forkhead box N3 on TC progression, clarifying the tumorigenic role of the Wnt/β-catenin signaling in TC [25]. In addition, deletion of β-catenin could mitigate the promotion effect of syndecan 4 on TC cell migration and invasion, revealing that activation of Wnt/β-catenin promotes the progression of TC [26]. It follows that inhibition of the Wnt/β-catenin signaling pathway might be a potential approach to the treatment of TC. However, whether the Wnt/β-catenin signaling pathway takes part in AHNAK2-mediated TC tumorigenesis is still unclear. In this paper, we investigate the association between AHNAK2 with the Wnt/β-catenin signaling and found that silencing of AHNAK2 blocked the activation of the Wnt/β-catenin signaling. More importantly, activation of the Wnt/β-catenin signaling by LiCl administration attenuated the inhibitory effect of AHNAK2 silencing on cell proliferation, metastasis, and EMT in TPC-1 cells. While, inhibition of the Wnt/β-catenin signaling by ICG-001 mitigated the promotion effect of AHNAK2 overexpression on cell proliferation, metastasis, and EMT in FTC-133 cells. Our data uncovered that silencing of AHNAK2 restricted the progression of TC by inducing the inactivation of the Wnt/β-catenin signaling, providing mechanistic evidence for the carcinogenic role of AHNAK2 in TC.

In conclusion, we found that AHNAK2 was upregulated in TC cells and its expression was tightly correlated with the pathological stage in TC. Our study also provides clinical evidence for the carcinogenic role of AHNAK2 in TC progression. Further, our findings uncovered that AHNAK2 exerted its carcinogenic role in TC via the Wnt/β-catenin pathway, and targeting AHNAK2 might be a novel and potential therapeutic strategy for TC.

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