

## Original Article

# High expression of HAX-1 protein is associated with tumor growth in papillary thyroid carcinoma

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**Abstract:** Papillary thyroid carcinoma (PTC) is the most common type of endocrine malignancy. HS1-associated protein X-1 (HAX-1) is an anti-apoptotic factor involved in the development of many types of cancer. However, its functional role in human PTC remains unclear. Here we investigated HAX-1 overexpression in human PTC samples and correlated with tumor size and TNM stage. Decreased expression of HAX-1 significantly inhibited proliferation, migration, and invasion in papillary thyroid cancer cell lines TPC1 and K1. Furthermore, we found that down-regulation of HAX-1 induced cell apoptosis. Our results suggest that HAX-1 plays a significant role in regulating the biological behavior in PTC cells and may contribute to PTC target therapy.

**Keywords:** HAX-1, papillary thyroid carcinoma, apoptosis, migration, invasion

## Introduction

PTC is the most common type of all thyroid cancers [1]. Despite most of the patients suffering from PTC having satisfactory long term prognosis with approximately 95% 5-year survival rate, about 20% of PTC patients exhibit distant metastasis or local recurrence [2]. Lymph node metastasis develops in the early stages of PTC and enhances the rate of mortality and recurrence [3, 4]. Mechanisms regarding tumorigenesis to therapeutic means remain unclear, indicating the need for more studies to identify novel treatments for PTC.

HCLS1-associated protein X-1 (HAX-1) was first considered as a protein, which contributes to B cell signaling transformation by interplaying with HS-1 (hematopoietic lineage cell-specific protein-1) [5]. HAX-1 can exert its function by interacting with diverse structural proteins, indicating its contribution in intracellular signaling [5-7]. HAX-1 is widely expressed in human and mouse tissues and has been suggested to be positioned in cell membranes and the endo-

plasmic reticulum (ER). HAX-1 has a plurality of functions including cell migration, invasion, and adhesion. In addition, HAX-1 participates in metastasis and tumorigenesis of a variety types of tumors [8]. Accumulating studies found that HAX-1 is overexpressed in multiple solid carcinomas, such as lung, breast, and hepatocellular carcinoma [9]. These results indicate that the dysfunction of HAX-1 contributes to tumorigenesis. HAX-1 is an effective anti-apoptotic protein of expression and is mainly observed in the mitochondria [9, 10]. HAX-1 has been found to bind with proteins regarding mitochondrial membrane permeability and the factors causing apoptosis [6, 10, 11].

Until now, there has been no research regarding the involvement of HAX-1 in PTC. In the present study, we analyzed HAX-1 expression in PTC tissues and the association between HAX-1 and clinical pathological features of PTC. Moreover, the function of HAX-1 in PTC and the potential pathogenesis contribution in PTC cell lines was also examined.

## Materials and methods

### *Tissue specimen acquisition*

This study was performed with the approval of the Ethics Committee of First Affiliated Hospital of China Medical University, and informed consent was obtained from all the patients with PTC whose tissue specimens were used in this study. None of the patients included suffered from other concurrent malignancies or were anaplastic or had other poorly differentiated follicular carcinomas of the thyroid or underwent any treatment prior to surgery. Fresh-frozen tumor tissues (n=12) and adjacent normal tissues (n=12) were obtained from patients with PTC undergoing surgical resection at the First Affiliated Hospital of China Medical University. Samples were stored at -80°C for further experiments. Paraffin section specimens from the thyroid were obtained from 102 Chinese patients who had PTC. All patients had a clinical duration of less than 3 years and had been admitted to the hospital for standard thyroidectomies between 2011 and 2013. Diagnoses were confirmed through histopathological examination. None of the patients had received radiotherapy or chemotherapy before undergoing surgical. TNM stage was assessed according to the tumor, node, and metastasis system classification proposed by the American Joint Committee on Cancer (7th edition).

### *Cell culture and transfection*

Human papillary thyroid cancer cell lines TPC-1 and K1 were kind gifts from Dr Wei Sun. TPC-1 was cultured Dulbecco's modified Eagle's medium (DMEM, Gibco, NY, USA) and 10% fetal bovine serum (FBS, Hyclone, UT, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM): Ham's F12: MCDB105 (2:1:1) plus 2 mM glutamine and 10% fetal bovine serum. All cells were cultured under 5% CO<sub>2</sub> atmosphere 37°C. The medium was changed 2 days and sub-cultured when the cell population density reached to 70-80% confluence. HAX-1- and HAX-1-RNAi-lentiviral vectors were purchased from Shanghai GeneChem Company (Shanghai, China). The HAX-1 sequence was 5'-AGCCCAAATCCTATTTCAA-3' and the shRNA control sequence was 5'-TTCTCCGAA-CGTGTCACGTTT-3'.

### *Western blot assay*

Tissues and cells were lysed in radioimmuno-precipitation (RIPA) buffer and total protein concentration was determined with a bicinchoninic acid (BCA) assay (Beyotime, China). Twenty micrograms of total protein were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were washed, blocked, and incubated sequentially with specific primary antibodies, namely: rabbit polyclonal anti-HAX1 (1:1000, Abcam, Cambridge, UK), rabbit monoclonal anti-Cleaved-caspase 3 (1:1500, Abcam, Cambridge, UK), rabbit monoclonal anti-cleaved-caspase 9 (1:1500, Abcam, Cambridge, UK), rabbit monoclonal anti-BAX (1:1500, Abcam, Cambridge, UK), anti-DR5 (1:500, Abcam, Cambridge, UK) mouse polyclonal anti-Tubulin (1:3000, Santa Cruz, USA). Incubation in primary antibodies was followed by goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (1:5000, Origene Co., Ltd. Beijing, China). The reactions were detected by enhanced chemiluminescence assay. Each experiment was performed in triplicate.

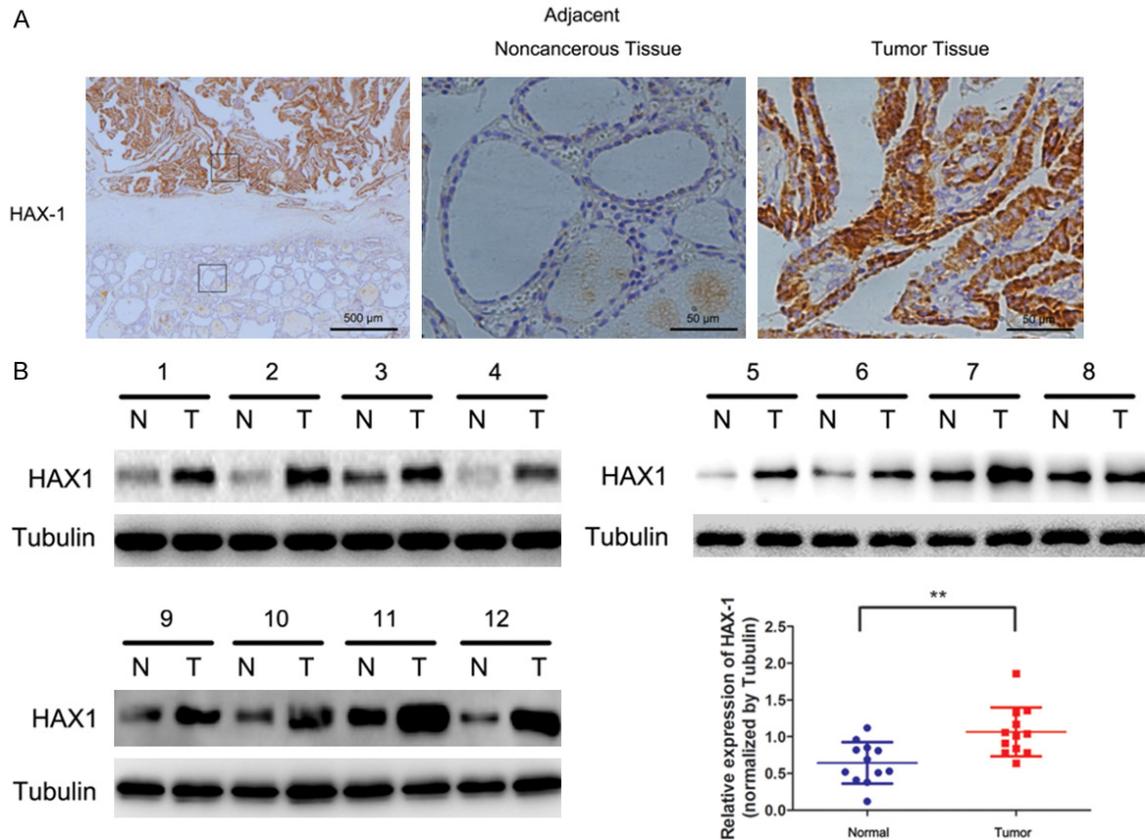
### *Cell viability assays*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cell viability. thyroid cancer cell were seeded on 96-well plates at 1 d, 2 d and 3 d after transfection, the samples were rinsed twice with sterile phosphate buffered saline (PBS) and the adherent cells were incubated in medium containing 100 mg/0.1 ml of MTT (Sigma Aldrich) for 4 h. After carefully removing the medium, the blue crystal layer attached to the surface of the material was then dissolved using dimethyl sulfoxide (DMSO). The optical density (OD) was measured at 490 nm using a spectrophotometer.

### *Cell migration assay and invasion assay*

Migration assays were performed using atranswell chamber with an 8-µm pore size for 24-well plates (Corning). Cells transfected with shHAX-1 and shNC were seeded in the upper chamber at 2-5×10<sup>5</sup> in DMEM serum free medium and DMEM with 10% FBS was added in the bottom of the chamber. After 24 h, the cells on the upper surface of the filter were removed carefully by a cotton swab. Chambers

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**Figure 1.** Expression of HAX1 in PTC tissue and adjacent non-cancerous tissue. A. Immunohistochemical staining of HAX-1 proteins in PTC tissues and adjacent non-cancerous tissues. B. HAX-1 levels was analyzed by Western blot assay in 12 PTC tissues and adjacent noncancerous tissues, and visualized via scatter diagram. \*\* $P < 0.01$ .

were fixed in 95% ethanol 15 min stained with 0.1% crystal violet. Three fields per chamber were randomly selected and the average cells numbers were counted. Cell invasion assays were performed in plates were coated by Matrigel (BD Bioscience, San Jose, CA, USA) and with similar conditions for the migration assay.

## Results

### *HAX-1 is upregulated in PTC and correlated with clinical characteristics*

To evaluate the role of HAX-1 in the progression of PTC, immunohistochemistry was used to test in 102 PTC specimens and their adjacent normal tissues (Figure 1A). The association between HAX-1 expression levels and patient clinical characters is shown in Table 1. High expression of HAX-1 was significantly associated with tumor size ( $P < 0.05$ ) and tumor TNM stage ( $P < 0.05$ ). No correlations were found

with gender, patient age, or lymph node metastasis. These findings suggest that levels and activity of HAX1 correlate with the stage of PTC. Furthermore, Western blot assay was employed for analysis of HAX-1 protein expression in 12 PTC tissues samples. As shown in Figure 1B, HAX-1 in PTC tissues was higher than that in the matched adjacent normal tissues ( $P < 0.01$ ).

### *Knockdown of HAX1 in PTC cell lines inhibits cellular proliferation and colony formation*

In order to characterize the role of HAX-1 in TPC1 and K1 cell proliferation, we first used a shRNA targeted to HAX1 (shHAX-1) markedly decreased the HAX-1 level compared to the control sequence (shNC) in both TPC1 and K1 cells (Figure 2A). Next, to assess cells proliferation rate we used MTT assay. The result showed that the proliferation rate of shHAX-1 group was significantly reduced than that of shNC group in both cell lines (Figure 2B). Furthermore, clonogenic assays showed that

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**Table 1.** Clinicopathologic features of 102 thyroid carcinomas patients

| Characteristics | Expression of hax1 |           | P value |
|-----------------|--------------------|-----------|---------|
|                 | Low                | High      |         |
|                 | Number=39          | Number=63 |         |
| Gender          |                    |           |         |
| Male            | 15                 | 35        | 0.093   |
| Female          | 24                 | 28        |         |
| Age (years)     |                    |           |         |
| <50             | 18                 | 30        | 0.885   |
| ≥50             | 21                 | 33        |         |
| LNM             |                    |           |         |
| Yes             | 17                 | 37        | 0.115   |
| No              | 22                 | 25        |         |
| TNM Stage       |                    |           |         |
| I+II            | 25                 | 25        | 0.017*  |
| III+IV          | 14                 | 38        |         |
| Tumor size (cm) |                    |           |         |
| <2              | 23                 | 23        | 0.027*  |
| ≥2              | 16                 | 40        |         |

LNM: lymph node metastasis. Statistical analyses were performed by the Pearson  $\chi^2$  test. \* $P < 0.05$  was considered significant.

HAX1 knockdown significantly inhibited cell colony formation (**Figure 2C**). These findings suggest that silencing HAX-1 could impair PTC cell growth.

### *Knockdown of HAX1 inhibits PTC cell migration and invasion*

To explore whether knockdown of HAX-1 could affect cell invasion and migration capacities, we performed transwell assay in both cell lines transfected with shHAX1 and shNC. The results showed that, compared to the shNC group, the migration and invasion cell number of TPC1 and K1 in shHAX1 group was dramatically down-regulated (**Figure 2D** and **2E**). Moreover, the results confirmed that down-regulation of HAX-1 could reduce PTC cell migration and invasion ability.

### *HAX-1 knockdown induced PTC cells apoptosis*

To further study the effect of HAX-1 inhibition on the apoptosis of PTC, cells transfected with shHAX-1 and their control group cells were stained with Annexin V and PI. The flow cytometry results suggested that the apoptosis induced by shHAX-1 was significantly increased in both TPC1 and K1 cell lines. In addition, the

expression levels of cleaved caspase 3 and 9 were dramatically increased through suppression of HAX-1 expression in PTC cells (**Figure 3B**). Moreover, we examined whether BAX and death receptor 5 (DR5) were regulated by HAX-1 silencing. The result showed that both BAX and DR5 were up-regulated in TPC and K1 cells.

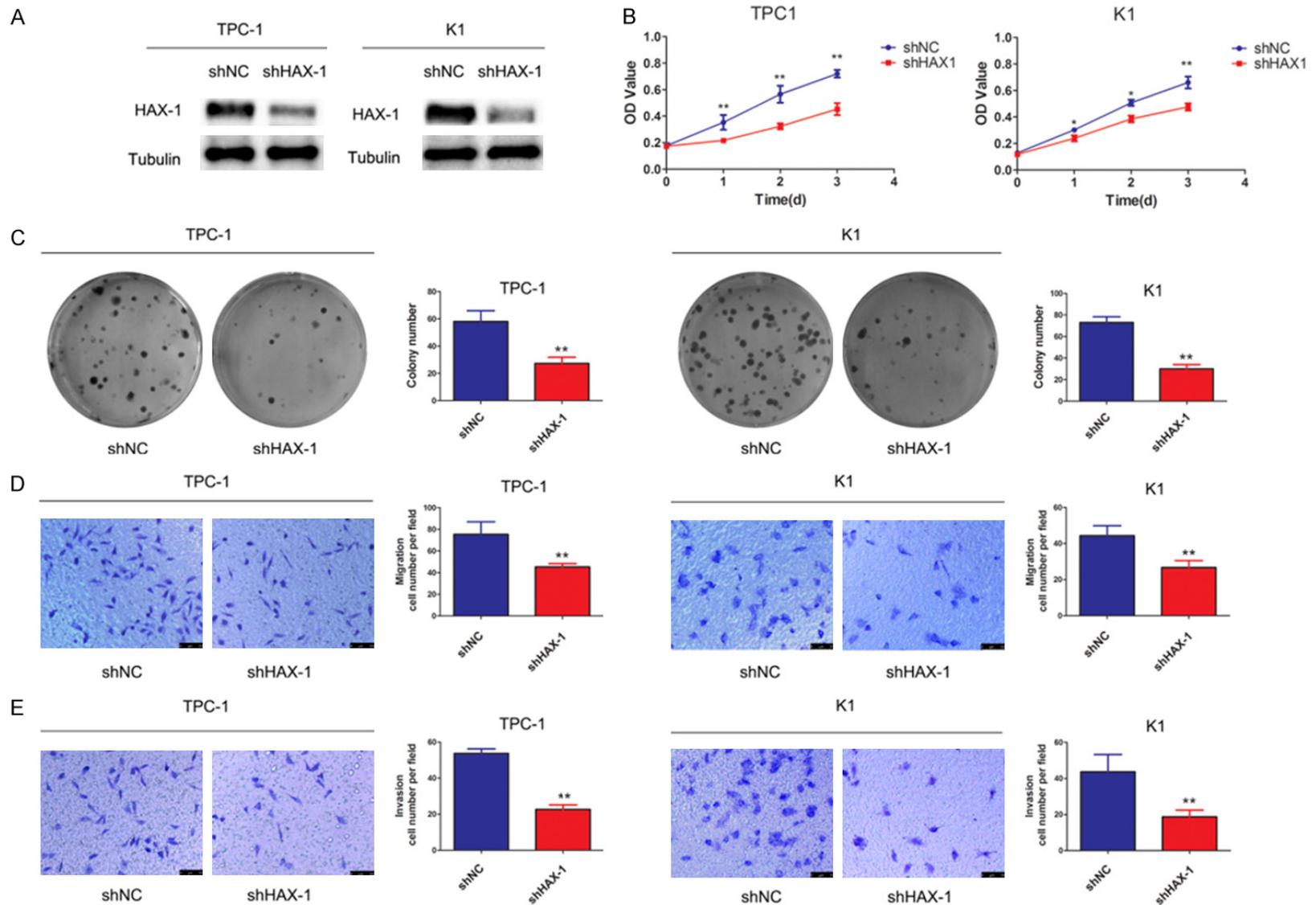
## Discussion

In the present study, our results shown that HAX-1 expression is up-regulated and correlated with clinical pathological features in PTC. Additionally, knockdown of HAX-1 in two PTC cell lines (K1 and TPC-1) inhibited proliferation, migration and invasion. In addition, HAX-1 also exhibited apoptosis in TPC-1 and K1 cell lines since repression of HAX-1 can caused cell apoptosis.

PTC is the most regular thyroid cancer in clinical symptoms. Most PTC has no obvious early symptoms, hence the unobvious symptom and the difficulties lying in the early diagnosis largely affect the effect of the treatment [12]. In this study, we found that expression of HAX-1 was up-regulated in human PTC samples compared to normal thyroids by Western blot and immunohistochemistry. On the basis of Oncomine [13], a cancer microarray database, HAX-1 was over-expressed in several types of carcinoma, such as hepatoma, lung cancer, myeloma, and lymphoma. HAX-1 plays a crucial role in carcinogenesis and has been demonstrated to be up-regulate in multiple tumor cell lines, which provides a strong evidence of its role in tumorigenesis and metastasis. In our results, there was a positive correlation between HAX-1 expression and TNM stage. HAX-1 was also closely associated with tumor size. These results are in accordance with the results of other carcinomas. Wang et al. reported that up-regulation of HAX-1 is positively correlated with cancer grade in hepatocellular carcinoma [14], suggesting that HAX-1 may contribute to the tumorigenesis and progression of PTC.

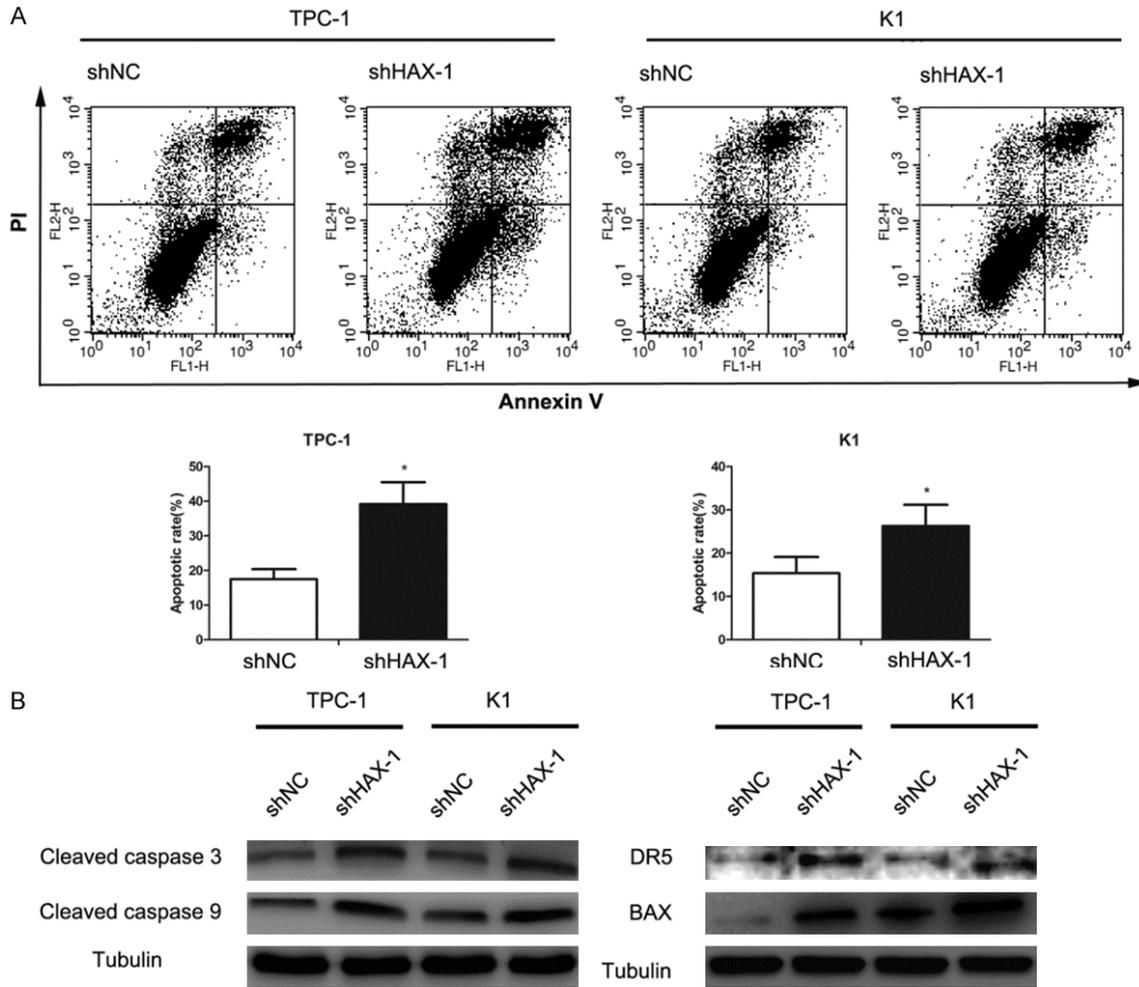
According to our *in vitro* study, silencing HAX-1 can remarkably repress cell proliferation, migration, and invasion in two PTC cell lines, TPC1 and K1. HAX-1 has been suggested to exert a vital role in the mediation of cell migration [7, 8]. The HAX-1 chaperone HS1 is isogenous to cortactin, which is a cytoskeletal protein mostly elevated in cancer [5]. Due to the homology

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**Figure 2.** Knockdown of HAX1 in PTC cell lines inhibits cellular proliferation, migration, and invasion. A. HAX-1 knockdown in TPC-1, and K1 cell lines, and the HAX-1 protein level was detected by western blot assay. Tubulin is presented as a loading control. B. Proliferation rate was evaluated at 1 day, 2 days, and 3 days through MTT assay. \* $P < 0.05$ . \*\* $P < 0.01$ . C. shHAX-1 was transfected into TPC-1 and K1 cell lines and the colony forming capacity was analyzed. \*\* $P < 0.01$ . D. HAX-1 knockout inhibits migration of TPC-1 and K1 cells. All data are presented as a mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$ . E. HAX-1 knockout inhibits invasion of TPC-1 and K1 cells. All data are presented as a mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$ .

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**Figure 3.** HAX1 knockdown induced PTC cells apoptosis. A. PI-AnnexinV staining show that HAX-1 inhibition in both TPC-1 and K1 cells significantly increased the percentage of apoptotic cells in the shHAX-1 group as compared to shNC group. \* $P < 0.05$ . B. Western blot analysis of the protein expression levels of cleaved caspase 3, cleaved caspase 9, DR5 and BAX in TPC-1 and K1 transfected with shHAX-1 and shNC.

between HS1 and cortactin [15], it is possible that HAX-1 can also bind with cortactin [8]. Several research studies had demonstrated that cortactin induces the invasion and metastasis in tumor cells [16]. HAX-1 can bind with cortactin by forming complex, thus promotes migration, while activates cell adhesion due to the lack of HAX-1 [11, 17]. A recent report has suggested that HAX-1 plays a vital role in the mediation of cancer cell migration and invasion by clathrin-regulated endocytosis [8]. Silencing of the HAX-1 gene significantly inhibited cell proliferation, migration, and invasion, which suggests that HAX-1 is involved in oncogenesis and development of PTC, which may repress differentiation and lymph node metastasis of this malignancy.

In our experiment, the expression of cleaved caspase 3 and 9 were also up-regulated when treating with shHAX-1. It has been previously reported that HAX-1 could repress caspase 9 activity. Moreover, it could induce accumulation of BAX (BCL2 associated X, apoptosis mediator) by interacting with HtrA2 (high temperature regulated A2), thus initiating the apoptotic pathway. HAX-1 may exert vital anti-apoptotic effects through modulating the mitochondrial pathway [5, 18-20]. It has been demonstrated that HAX-1 could prevent cell from mitochondrial impairment caused by environmental and other factors and reduce secretion of pro-apoptotic signals from the mitochondria membrane [21, 22]. In this study, BAX was increased through silencing HAX-1, and the apoptotic cell

death was highly increased as well. Consistent with our studies, it has been suggested that high expression of BAX was present in human PTC tissues and cell lines. Functional inhibition of HAX-1 increases apoptosis in two PTC cell lines. We also examined whether the expression of death receptor, DR5 was influenced by shHAX-1 supplement by Western blot. This result proves that elevated expression of DR5 by shHAX-1 treatment may contribute to cell death in PTC cell lines. This study also analyzed the cleavage of caspase-3 and 9 in response to treat with shHAX-1, in accordance with a role of apoptosis in HAX-1 regulated cell death. In addition, we measured expression by Western blot of DR5, a death receptor by silencing HAX-1 gene, in two PTC cell lines. Cell death caused by apoptosis is initiated by co-action between death receptor 5 and knockdown HAX-1 significantly increased protein expression of DR5 in both cell lines. Apoptosis is a vital mediator of tumorigenesis, which may be promoted by activation of agonists such as the tumor necrosis factor (TNF) family [23]. TNF-related apoptosis inducing ligand (TRAIL) is a potential death receptor (DR) ligands leading to apoptosis via binding with DR in multiple carcinoma cell lines [24, 25]. This combination results in the consequent activation of caspase 9, which mediates downstream caspases activation and expression of pro-apoptotic protein BAX. The role of DR signaling pathway during tumor initiation and malignant progression has made it an important regulator in cancer treatment.

### Conclusion

Our results suggest that HAX-1 is over-expressed in PTC tissues and that up-regulation of HAX-1 is related to histological grade, as well as tumor size. Moreover, silencing HAX-1 promoted apoptosis, while inhibiting cell proliferation, migration and invasion. Our study provides novel insight that HAX-1 may function as a new orientation for PTC therapy.

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### Disclosure of conflict of interest

None.

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