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ORIGINAL ARTICLE

# VASN promotes proliferation of laryngeal cancer cells via YAP/TAZ

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## Summary

***Purpose:*** *To explore whether vasorin protein (VASN) can affect the proliferation of laryngeal cancer cells through the regulation of yes-associated protein (YAP)/TAZ (transcriptional co-activator with PDZ binding motif), and then promote the development of laryngeal cancer.*

***Methods:*** *Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of VASN in laryngeal carcinoma tissues and different T-stage tumor patients, and the correlation between VASN expression and clinicopathological features was analyzed. The diagnostic value of VASN for laryngeal cancer was assessed by receiver operating characteristic (ROC) analysis. Kaplan-Meier method was used to plot the survival curves of patients with different VASN expression levels. After knocking down VASN in Hep-2 cells or in overexpressing VASN in TU212 cells, cell viability, proliferation ability and protein expression level of YAP/TAZ were detected by cell counting kit-8 (CCK-8), plate cloning assay and Western blot. Furthermore, YAP was overexpressed or knocked down simultaneously to evaluate its effect on the viability and proliferation ability of cells.*

***Results:*** *The expression of VASN in laryngeal carcinoma was significantly higher than that in the normal control group, while, at the same time, the expression of VASN in the t3+t4 tumor patients was significantly higher than that in the t1+t2 tumors. We also found that the expression level of VASN was closely related to N stage, T stage, and lymph node metastasis, suggesting that VASN had a certain diagnostic value for laryngeal cancer. After knocking down VASN in cells, the cell viability, proliferative capacity and YAP/TAZ protein expression level decreased significantly. Besides, overexpressing YAP could reverse the inhibition of cell viability and proliferation ability caused by VASN knockdown.*

***Conclusions:*** *VASN can promote the development of laryngeal cancer by affecting the expression of YAP/TAZ.*

***Key words:*** *laryngeal cancer, VASN, YAP/TAZ*

## Introduction

Laryngeal cancer is one of the common malignant tumors of the head and neck. It is also a relatively invasive malignant tumor, accounting for 2.4% of new tumors each year. Its main causes are bad living habits, external environment, genetic factors and human papillomavirus (HPV) infection [1]. The clinical treatment of laryngeal cancer mainly includes surgery, radiotherapy and chemotherapy. Although the surgical techniques and comprehensive treatment plans are continuously optimized, the toxic side effects of treatment seriously affect the patient quality of life, making its prognosis not satisfactory. Currently, there is no effective targeted drug for laryngeal cancer [2,3]. Therefore, the search for ideal and effective molecular markers is of great importance for improving the clinical diagnosis and treatment of this disease.

Vasorin protein (VASN) is a type I transmembrane protein with tandem leucine domain, epidermal growth factor domain and fibronectin III

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domain [4]. It can be cleaved by the metalloproteinase ad-am17 and detached from the cell membrane to produce a soluble VASN protein. Human VASN protein is mainly expressed in the aorta, kidney and placental tissues, and the expression levels in other tissues and organs is extremely low [5]. A study [6] found that VASN is highly expressed in liver cancer cells and liver cancer tissues, and can be used as a potential biomarker for liver cancer. VASN also exists in exosomes derived from HepG2 cells to promote migration of HUVEC cells [7]. However, there is no correlation study between VASN and laryngeal cancer.

Yes-associated protein (YAP)/TAZ (transcriptional co-activator with PDZ binding motif) is a homologous protein and is the most important transcriptional coactivator in the Hippo pathway. YAP/TAZ is involved in many physiological and pathological processes, including regulation of organ volume, cell proliferation, differentiation, apoptosis, epithelial-mesenchymal transition, and cell contact inhibition. It has been confirmed that YAP/TAZ participates in embryo development [8], stem cell proliferation, differentiation [9,10], vascular remodeling of cardiovascular disease [11], progress in neurodegenerative diseases [12-16], tumor occurrence, development, drug resistance and prognosis [17,18] and so on. However, there have been no reports of research between YAP/TAZ and laryngeal cancer. A recent study has shown that VASN promotes thyroid carcinogenesis *in vitro* via the YAP/TAZ pathway [19]. Therefore, in the present study we explored whether VASN can affect the proliferation of laryngeal cancer cells through regulation of YAP/TAZ, hoping to provide new ideas for the clinical diagnosis and treatment of laryngeal cancer.

## Methods

*Clinical samples*

A total of 58 patients with laryngeal cancer were enrolled in the study. The surgically resected laryngeal cancer tissue specimen formed the laryngeal cancer group, and the normal laryngeal tissue specimen away from the tumor formed the control group (paracarcinoma tissues). No patient was treated with chemoradiotherapy. All tissues were quickly frozen in liquid nitrogen immediately after intraoperative resection, and then stored at -80°C. The patients were informed of the study, signed the informed consent form and the study was approved by the hospital Ethics Committee.

*Cell culture*

The laryngeal cancer cell lines Hep-2, TU212, M4E and M2E and normal epithelial cell line NP69 were purchased from Shanghai Chinese Academy of Sciences (Shanghai, China). All samples were cultured in Dulbecco's Modified Eagle's Medium (DMEM), high glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C and 5% $CO_2$.

*RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) detection*

Total RNA in tissues and cells was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), and the expression level of VASN was detected using a PCR detection kit. The primer sequences were as follows: VASN, forward 5'-CCACCTGCCCTTTGTCCTG-3' and reverse 5'-CAACCTGCCGCTCCTCATT-3'; glyceraldheyde 3-phosphate dehydrogenase (GAPDH) forward 5'-CAATGACCCCTTCATTGACC-3', and reverse 5'-GACAAGCTTCCCGTTCTCAG-3'.

*Cell transfection*

Cells in the logarithmic growth phase were selected. When the cell density reached 80% in 6-well cell culture plates, 100 μmol/L of si-VASN was transfected into cells with 10 μL of Lipofectamine 2000 (diluted with OPTI-MEM) (Invitrogen, Carlsbad, CA, USA). After 6 h, the medium was replaced by DMEM high sugar medium containing 10% FBS. (si-VASN, forward 5'-GCAUGAAAUCACCAAUGAGTT-3', reverse 5'-CUCAUUGGUGAUUUCAUGCTT-3'; si-YAP forward 5'-GCGUAGCCAGUUACCAACATT-3', reverse 5'-UGUUGGUAACUGGCUACGCTT-3').

*Cell counting kit-8 (CCK-8) assay*

The cells were digested and seeded in 96-well plates at $3\times10^3$ cells/well in 200 μL of medium, and the viability of the cells at 6, 24, 48, 72 and 96 h was measured, respectively. At each detection time point, 10 μL of 10% CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well, and then the cells were cultured in an incubator at 37°C and 5% $CO_2$ for 3 h. The optical density (OD) value of each well was detected by a microplate reader at a wavelength of 450 nm. The growth curve was plotted with time as the abscissa and OD as the ordinate.

*Plate cloning experiment*

$5\times10^2$ LSCC cells were seeded into each well of 6-well plates. The liquid in the 6-well plates was gently shaken and placed in a 37°C incubator. After 24 h, the DMEM was changed. After 14 days, the medium was discarded and the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

*Western blot assay*

The collected cells were washed with phosphate buffered saline (PBS), and lysed with radioprecipitation assay (RIPA) and the supernatant was collected for 30 min and placed in an ice bath to determine the protein concentration. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 5% skim milk powder for 1 h at room temperature. Primary antibodies were added for incubation

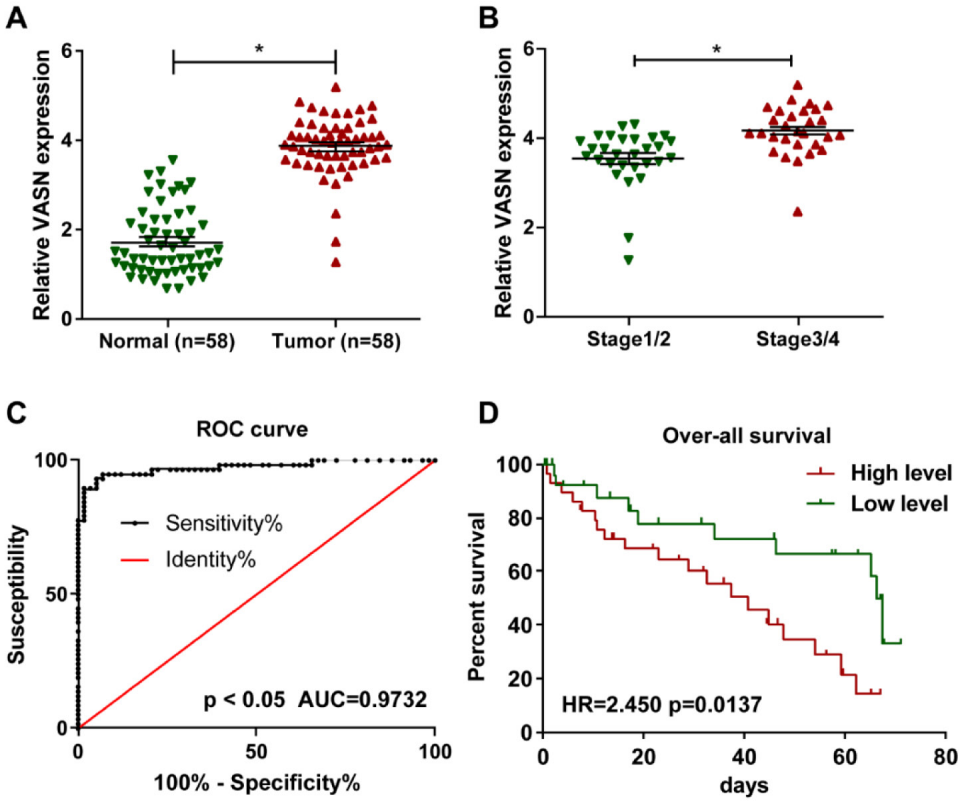


**Figure 1.** VASN is highly expressed in laryngeal cancer. **A:** qRT-PCR results showed that the expression of VASN in 58 laryngeal carcinoma tissues was significantly higher than that in the normal control group (*p<0.01). **B:** qRT-PCR was used to detect that the expression of VASN in T3+T4 tumor patients (*p<0.05). **C:** ROC curve between VASN expression and diagnostic sensitivity of laryngeal cancer. **D:** The overall survival rate of patients with high expression of VASN in laryngeal cancer was significantly lower than that in the low expression group of VASN.

**Table 1.** Demographic and clinicopathologic features in High and Low VASN expression groups of patients with laryngeal cancer (n=58)

| *Clinicopathologic features* | *Number of cases* | *VASN expression* | | *p value* |
|---|---|---|---|---|
| | | *Low (n=29)* | *High (n=29)* | |
| Age (years) | | | | 0.7924 |
| ≤65 | 31 | 15 | 16 | |
| >65 | 27 | 14 | 13 | |
| Gender | | | | 0.5975 |
| Male | 26 | 12 | 14 | |
| Female | 32 | 17 | 15 | |
| N stage | | | | 0.0178 |
| N0 | 31 | 20 | 11 | |
| N1, N2, N3 | 27 | 9 | 18 | |
| T stage | | | | 0.0205 |
| Stage 1/2 | 30 | 11 | 19 | |
| Stage 3/4 | 28 | 18 | 10 | |
| Lymph node metastasis | | | | 0.0170 |
| Absent | 33 | 21 | 12 | |
| Present | 25 | 8 | 17 | |

overnight at a 4°C shaker. In the next day, the membrane was rinsed 3 times with tris buffered saline-tween (TBST) and incubated with second antibody for 1 h at room temperature. After that, the protein samples on the membrane were finally semi-quantitatively analyzed by alpha SP image analysis software.

*Statistics*

Statistical analyses were performed using SPSS 22.0 statistical software (IBM, Armonk, NY, USA). The measurement data were analyzed by independent sample t-test; the count data was tested by $x^2$. The receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of VASN for laryngeal cancer. Survival curves were plotted using the Kaplan-Meier method and the difference in patient survival was compared using the Log-rank test. The difference was statistically significant at $p<0.05$.

## Results

*VASN is highly expressed in laryngeal cancer*

qRT-PCR showed that the expression of VASN in 58 laryngeal carcinoma tissues was significantly higher than that in the normal control group (Figure 1A); meanwhile, the expression of VASN in T3+T4 tumor patients was significantly higher than that in T1+T2 tumors (Figure 1B). The ROC curve

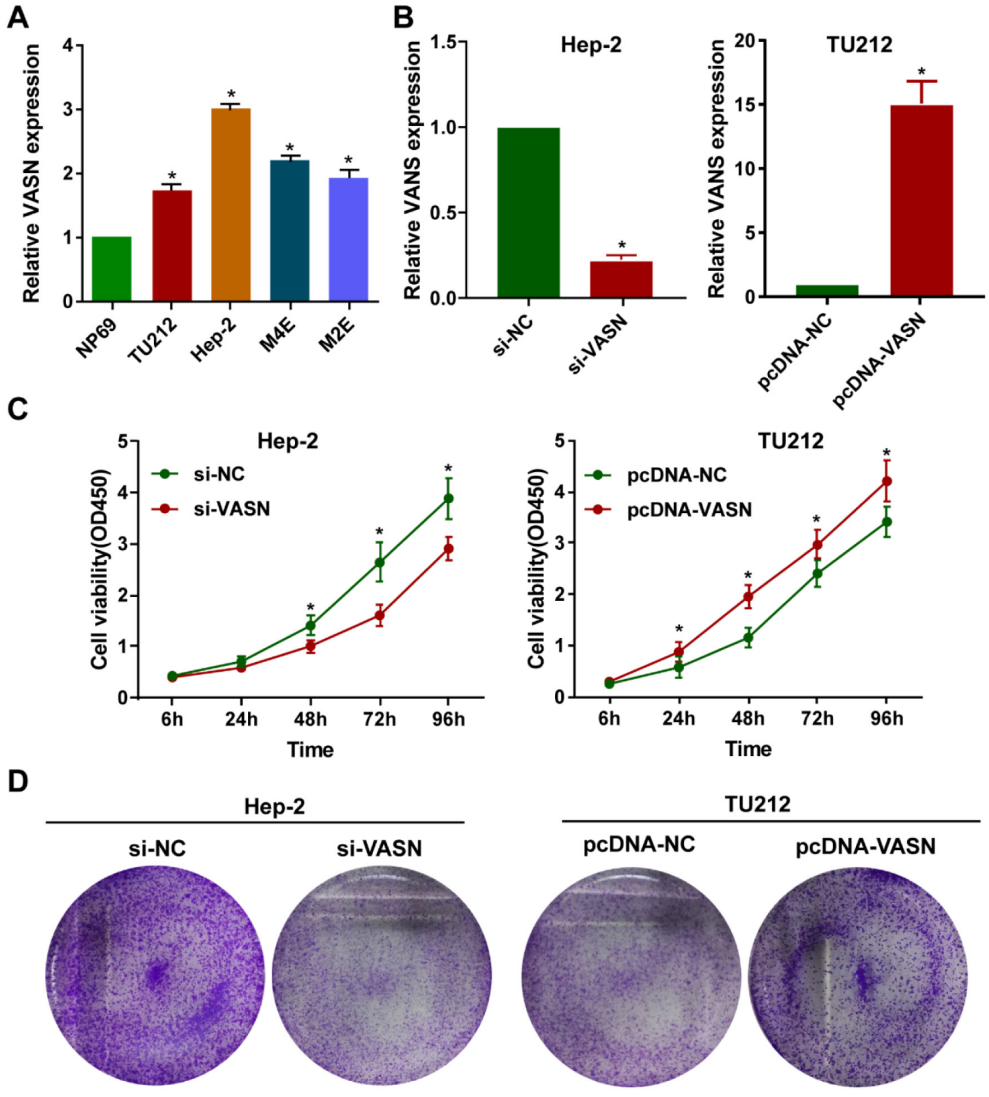


**Figure 2.** Overexpression of VASN promotes cell proliferation. **A:** qRT-PCR showed that the expression of VASN in laryngeal carcinoma cell lines Hep-2, TU212, M4E and M2E was significantly higher than that of normal epithelial cells NP69 (compared with NP69, *p<0.05). **B:** After knockdown of VASN in Hep-2 cells or overexpression of VASN in TU212 cells, VASN expression was detected by qRT-PCR (compared with si-NC or pcDNA-NC, *p<0.05). **C:** After knocking down VASN in Hep-2 cells, CCK-8 showed that the cell viability was significantly decreased; after simultaneous overexpression of VASN in TU212 cells, the above result was reversed (compared with 6h, *p<0.05). **D:** After knocking down VASN in Hep-2 cells, the cell proliferation ability via cell cloning assay was significantly decreased; after simultaneous overexpression of VASN in TU212 cells, the above result was reversed. (magnification: 40×)

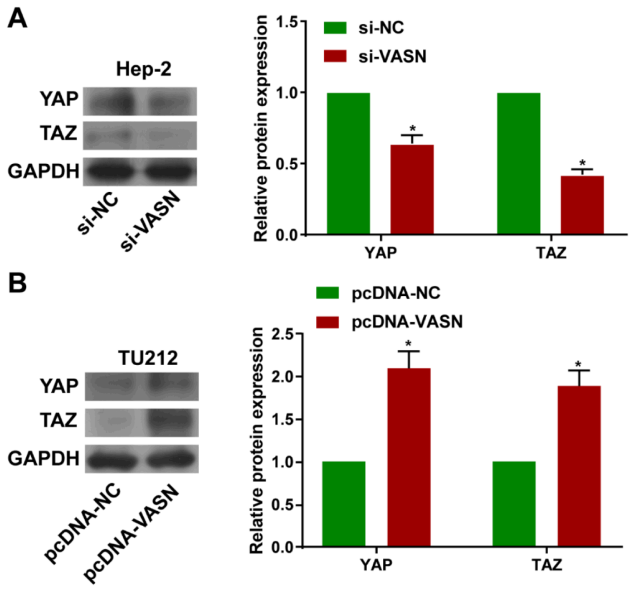


**Figure 3.** YAP/TAZ expression is inhibited after knockdown of VASN. **A:** Western blot revealed that after knocking down VASN in Hep-2 cells, the protein expression level of YAP/TAZ was significantly decreased (compared with si-NC, *p<0.05). **B:** Western blot revealed that after overexpressing VASN in TU212 cells, the protein expression level of YAP/TAZ was significantly higher (compared with pcDNA-NC, *p<0.05).

between the expression of VASN and the diagnostic sensitivity of laryngeal cancer showed that the area under the curve (AUC) was 0.9732, p<0.05 (Figure 1C). According to the analysis of survival curves, the overall survival rate of patients with high expression of VASN was significantly lower than that of VASN low expression group, (HR=2.450, p=0.0137) (Figure 1D). The above results indicated that VASN was associated with the development of laryngeal carcinoma, and had the potential to be a marker for laryngeal cancer.

*Correlation between expression of VASN and clinicopathological features in patients with laryngeal cancer*

In order to clarify the relationship between VASN and clinicopathological factors in patients with laryngeal cancer, we used qRT-PCR to detect the median relative expression of VASN in this disease, and divided the patients into 29 cases with high expression and 29 cases with low expression. The results showed that the expression level of VASN was statistically different in different T stage, N stage and lymph node metastasis (p<0.05), but there was no statistical difference in age and gender (p>0.05) (Table 1). The above results indicated that the expression level of VASN was associated with T stage, N stage and lymph node metastasis.

*Overexpressing VASN could promote cell proliferation*

VASN expression in laryngeal carcinoma cell lines Hep-2, TU212, M4E and M2E was significantly higher than that of normal epithelial cells NP69, with Hep-2 expression being the highest and lowest in TU212 (Figure 2A). Therefore, in this study we used Hep-2 and TU212 cells for subsequent experiments. VASN was overexpressed and knocked down in Hep-2 cells, and the transfection efficiency was detected (Figure 2B). After knocking down VASN in Hep-2 cells, the cell viability and cell proliferation ability of CCK8 and were significantly decreased. Besides, when VASN was overexpressed in TU212 cells, the results were reversed (Figure 2C,2D). The above results revealed that VASN could affect the viability and proliferation of cells, exerting a promoting effect.

*Knocking down VASN could suppress YAP/TAZ expression*

To explore the relationship between VASN and YAP/TAZ, we knocked down VASN in Hep-2 cells and found that the protein expression level of YAP/TAZ was significantly decreased by Western blot (Figure 3A); meanwhile, VASN overexpression in TU212 cells could upregulate the level of YAP/TAZ (Figure 3B). These results showed that VASN could promote the expression of YAP/TAZ.

*Overexpression of YAP reverse knockdown of VASN inhibits cell proliferation*

To further clarify the related mechanisms of VASN, YAP/TAZ and laryngeal carcinogenesis, we overexpressed YAP in Hep-2 cells that had knocked down VASN, and found that the inhibition of cell viability by VASN was reversed. Meanwhile, we found that knockdown of YAP in TU212 cells reversed the promotion of cell viability caused by VASN overexpression (Figure 4A). In addition, overexpression of YAP in Hep-2 cells reversed the inhibition of cell proliferation by knockdown of VASN; at the same time, knockdown of YAP in TU212 cells reversed the promotion of cell proliferation caused by overexpression of VASN (Figure 4B). The above results suggested that VASN could promote cell viability and proliferation ability through YAP/TAZ.

## Discussion

Laryngeal cancer is one of the most common malignant tumors in the upper respiratory tract [20]. The 5-year survival rate of patients with laryngeal cancer is about 60%. Although progress has been made in the diagnosis and treatment of laryngeal cancer, there is no significant improvement

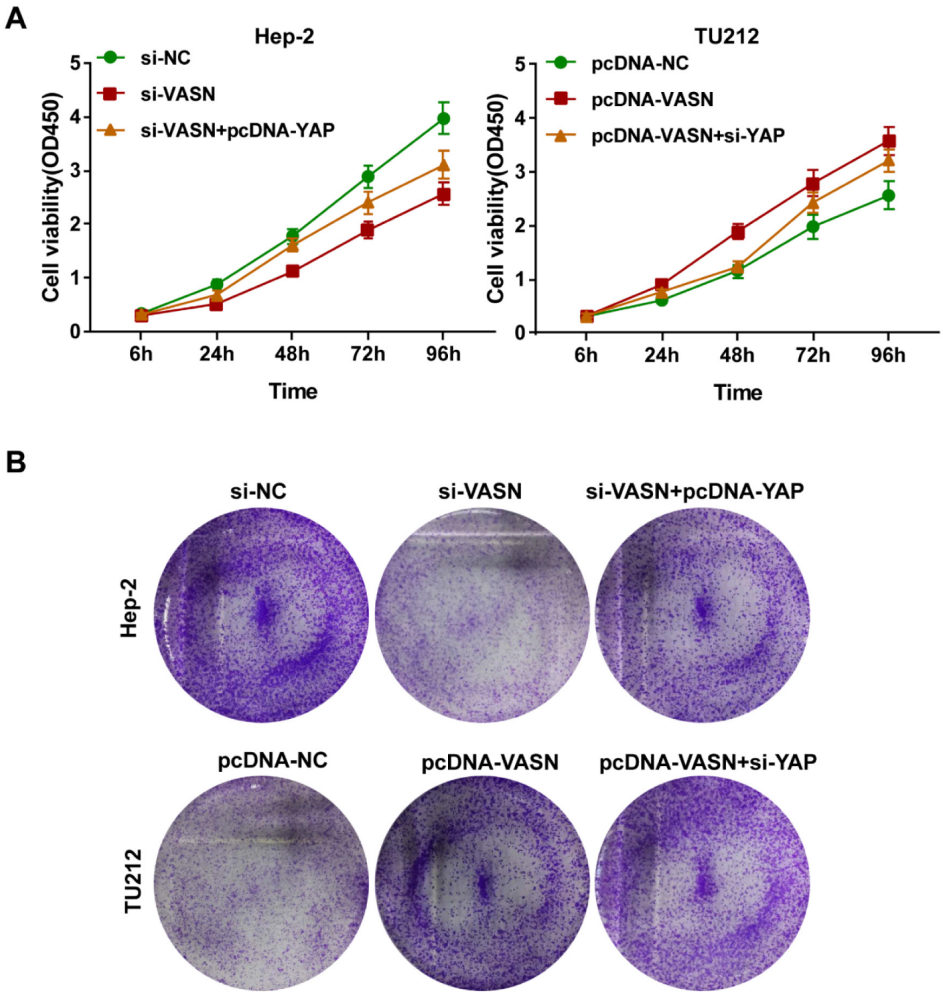


**Figure 4.** Overexpression of YAP reverses the inhibitory effect of knockdown of VASN on cell proliferation. **A:** Overexpression of YAP in Hep-2 cells reversed the inhibitory effect of knockdown of VASN on cell viability; knockdown of YAP in TU212 cells reversed the effect of overexpression of VASN on cell viability. **B:** Overexpression of YAP in Hep-2 cells reversed the inhibitory effect of knockdown of VASN on cell proliferation; knockdown of YAP in TU212 cells reversed the effect of overexpression of VASN on cell proliferation. (magnification: 40×)

in the survival rate and mortality [21]. Cancer cell invasion, recurrence and metastasis are important causes of surgical failure and death [22]. Therefore, the identification and research of new molecular markers during the development and progression of laryngeal cancer are of great importance for improving the clinical prognosis of patients with this disease.

The VASN protein is a newly discovered protein molecule. Studies have shown that although human VASN protein is expressed only in a few normal tissues, it is highly expressed in several tumor cells and tumor tissues. VASN antagonizes TGFβ and promotes vascular endothelial cell migration, suggesting that it may be related to tumorigenesis and disease development [6,23]. Choksi et al [24] showed that hepatocytes from VASN knocked out mice were significantly more sensitive to TNFα-induced apoptosis, whereas human glioma cells knocked out of VASN showed increased sensitivity to hypoxia-induced apoptosis. These results suggest that VASN may be involved in the cellular stress response process. Other studies have shown that VASN is highly expressed in hepatocytes and may serve as a medium for information exchange between HepG2 and HUVEC cells [7]. In the present study, we found that VASN was highly expressed in laryngeal cancer tissues and may have a role in promoting the development of laryngeal carcinoma. In addition, the results of the ROC curve analysis suggested that VASN might serve as a potential biomarker for the diagnosis of laryngeal cancer.

In mammals, YAP and TAZ are downstream effector molecules of the Hippo pathway. The YAP gene is localized on the human chromosome 11q22 and is widely expressed in body tissues under normal physiological conditions.TAZ is also known as

WWTR1, which is homologous to YAP, and 46% of the amino acid sequences are identical [25]. YAP and TAZ are transcriptional coactivator of shuttle between cytoplasm and nucleus. When Hippo kinase module is in activated state, LATS1/2 and MST1/2 will phosphorylate, inactivate YAP and TAZ, and promote cell apoptosis, thereby inhibiting cell growth. Conversely, when the kinase module is suppressed, LATS1/2, MST1/2 phosphorylation of YAP, TAZ is inhibited, and low-phosphorylated YAP and TAZ can be transferred to the nucleus, raising nuclear YAP and TAZ, thereby inhibiting cells apoptosis and promoting epithelial-mesenchymal transition and tumor formation [26]. Bhandari et al [19] have shown that VASN promotes the development of thyroid cancer by inducing the carcinogenic YAP/TAZ pathway. Similar to this conclusion, in our stsudy, we found that VASN can affect the expression of YAP/TAZ, and overexpressed YAP can reverse the inhibitory effect of VASN on cell viability and proliferation. We showed that VASN can promote the viability and proliferation of cells through YAP/TAZ, which in turn affected the development of laryngeal cancer.

## Conclusions

In summary, VASN is highly expressed in laryngeal carcinoma tissues, and its expression level is related to the T stage, N stage and lymph node metastasis in laryngeal cancer patients. VASN can up-regulate the expression of YAP/TAZ and promote the proliferation of laryngeal cancer cells. Our study can provide a new idea for the diagnosis and treatment of laryngeal cancer.

## Conflict of interests

The authors declare no conflict of interests.

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