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Hypoxia-induced HIF-1α/VASN promotes bladder cancer progression

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Hypoxia-inducible factor-1 α (HIF-1α) is important in regulating the hypoxia adaptive response of bladder cancer. Vasorin (VASN) is closely related to tumor development. However, the role of VASN in hypoxia-induced bladder cancer remains to be clarified. To establish the hypoxia model, low-grade bladder cancer cell line RT4 was cultured under hypoxic conditions. RT4 cells were transfected with small interfering RNA to inhibit HIF-1α and VASN expression, and transfected with VASN overexpression plasmid to increase VASN expression. Wound healing and transwell assays were used to assess cell migration. Western blot was performed to detect epithelial-mesenchymal transformation (EMT)-related proteins, and YAP/TAZ and PTEN/AKT pathways expression. We found that VASN was increased in bladder cancer tissues and cell lines (RT4 and T24). Hypoxia promoted low-grade bladder cancer cell migration and EMT progression. Furthermore, the level of VASN was up-regulated under hypoxia in RT4 cells. Functional experiments revealed that hypoxia-induced bladder cancer cell migration through up-regulating VASN. Besides, VASN regulated the YAP/TAZ and PTEN/AKT pathways. Notably, VASN expression was positively correlated with HIF-1α expression. HIF-1α activated VASN expression in hypoxia-induced RT4 cells. Therefore, our findings support the first direct evidence that VASN participates in the adaptive response to hypoxia in bladder cancer, which highlights VASN as a potential target for bladder cancer.

Keywords Bladder cancer, hypoxia, VASN, HIF-1α, EMT

Bladder cancer is one of the most common malignant tumors in the urinary system, with an annual 82,290 new cases and 16,710 deaths in the United States in 2023[1,2]. According to the aggressiveness of tumor cells, bladder cancer is divided into two types including non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC)[3]. At present, the clinical treatment options for bladder cancer mainly include surgical intervention, chemotherapy, radiotherapy, and immunotherapy[4,5]. However, bladder cancer has the characteristics of easy recurrence, easy progression and early metastasis, resulting in poor prognosis for patients[6]. Therefore, it is of great significance to study the biological mechanism of the occurrence and development of bladder cancer, especially in finding new therapeutic targets.

Numerous studies have shown that epithelial-mesenchymal transformation (EMT) plays a crucial role in the progression of bladder cancer[7,8]. Decreased epithelial cell marker (E-cadherin) and elevated levels of interstitial cell markers (N-cadherin and Vimentin) are key features of EMT. E-cadherin is a calcium-dependent transmembrane glycoprotein mainly distributed at the cell-cell contact site, which plays an important role in the maintenance of intercellular adhesion and the stability of extracellular matrix components[9]. During EMT, epithelial cells lose their tight connections and intercellular adhesion, which induces cancer cell migration, invasion, and metastasis[10–12]. In addition, EMT has also been associated with tumor drug resistance and the formation of tumor stem cells[13,14]. There are many interaction mechanisms between tumor-associated macrophages and EMT-favoring tumor cells, and a vicious cycle is formed to promote tumor invasion and metastasis[15]. Therefore, understanding the molecular mechanism regulating EMT process has become the key in the study of tumor metastasis mechanism.

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Hypoxia is an important characteristic of solid tumor microenvironment[16]. Hypoxia is associated with poor prognosis of tumors and is also an important marker of malignant tumors[16,17]. Hypoxia-inducible factor (HIF) plays an important role in regulating the hypoxia adaptive response of bladder cancer[18]. HIF-1α is one of the constituent subunits of HIF-1, which is one of the most important regulatory factors in hypoxic microenvironment[18]. Therefore, exploring the related mechanisms of signal pathway activation in hypoxic microenvironment are still important for finding new clinical therapeutic targets for bladder cancer.

Vasorin (VASN) is a typical type I transmembrane glycoprotein[19]. Several studies have reported that VASN is expressed in a variety of solid tumors, which is closely related to the malignant degree of tumors[20,21]. For example, VASN was up-regulated in serum-derived exosomes and tissues in patients with liver cancer and was a promising target for liver cancer diagnosis and treatment[20]. In addition, colorectal cancer patients with high VASN expression are at a higher risk of developing lung metastasis and adjuvant chemotherapy resistance[21]. Furthermore, the abnormally expressed VASN is involved in a variety of biological processes and plays an important role in the development of tumors[22,23]. For instance, VASN promoted the proliferation process of colorectal cancer and prostate cancer through the YAP/TAZ axis[22,23]. However, until now, the role of VASN in bladder cancer remains unclear.

The study aimed to explore the biological function of VASN in bladder cancer in hypoxic settings. In this study, we found that VASN was increased in high-grade bladder cancer tissues and cells. In addition, hypoxia-induced bladder cancer cell migration through up-regulating HIF-1α/VASN axis.

Materials and methods

Human samples

NMIBC and MIBC tissues were collected from patients undergoing surgery at The Affiliated Suqian First People's Hospital of Nanjing Medical University. Non-tumor tissues from each patient were obtained not less than 3 cm away from the edge of tumor tissue and served as the control group. None of these patients received preoperative radiotherapy and chemotherapy before the surgery. The consent agreement was signed by all participants and the project was approved by the Ethics Committee of The Affiliated Suqian First People's Hospital of Nanjing Medical University (approval number: 2023-SL-0054).

Cell culture and treatment

Human ureteral epithelial cell line (SV-HUC-1) and bladder cancer cell lines (RT4 and T24) were purchased from the American Type Culture Collection (ATCC, USA). SV-HUC-1 cells were cultured in F-12 K Medium (ATCC, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at 37℃ under normoxic conditions (20% O_2, 5% CO_2, 75% N_2). RT4 and T24 cells were maintained in DMEM (Gibco, USA) and RPMI 1640 medium (Gibco, USA) with 10% FBS at 37℃ under normoxic conditions (20% O_2, 5% CO_2, 75% N_2).

RT4 cells were chosen as a representative example of non-invasive bladder cancer cells. To establish the hypoxia model, low-grade bladder cancer cell line RT4 was cultured under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) at 37 °C for 48 h.

To observe the time-dependent changes in HIF-1α and VASN expression, RT4 cells were incubated under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) at 37 °C for 12, 24, 36, and 48 h.

Cell transfection

To knockdown VASN and HIF-1α expression, small interfering RNA against VASN (si-VASN) and small interfering RNA against HIF-1α (si-HIF-1α) were synthesized by Genepharma (Shanghai, China) and sh-NC was used as control. To overexpress VASN expression, the pcDNA3.1 vector was used to construct the VASN overexpression plasmid (VASN OE). For cell transfection, RT4 cells were transfected with the siRNA sequences and plasmids using Lipofectamine 2000 reagent (Invitrogen, USA).

Wound healing

RT4 and T24 cells were seeded into 6-well plates and incubated at 37℃ under hypoxic (1% O_2, 5% CO_2, 94% N_2) or normoxic conditions (20% O_2, 5% CO_2, 75% N_2) for 48 h. Then, a line was scraped into the cell monolayer using a sterile tip. The cells were washed three times with PBS to remove scratched cell debris, and then added to serum-free or low-serum medium at 37℃ under normoxic conditions (20% O_2, 5% CO_2, 75% N_2). After 24 h, the cell migration in the scratched area was observed with a microscope (Olympus, Japan) and photographed.

Transwell

For the transwell experiment, after the 48 h hypoxic or normoxic treatment, RT4 and T24 cells were cultured in serum-free medium in a transwell upper chamber with an 8 μm pore size. The cell medium containing 20% FBS was added in the transwell lower chamber. The incubation was carried out for 24 h under normoxic conditions. Then, the migrated cells were fixed in 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet. The migrated cells were counted by a light microscope (Olympus, Japan).

Hematoxylin and Eosin (HE) staining

NMIBC, MIBC and non-tumor tissues were fixed in 4% paraformaldehyde at room temperature for 24 h, followed by wax infiltration, embedding, and sectioning into 4 μm slices. The sections were de-paraffinized twice in xylene for 10 min each, then re-hydrated through a series of graded alcohols (100%, 95%, 90%, 80%, 70%) for 5 min each. Next, they were stained with hematoxylin (Servicebio, China) for 5 min and stained with 1% eosin solution for 2 min. Finally, representative images were taken using a light microscope (Olympus, Japan).

Immunohistochemical (IHC)

The paraffin-embedded tumor sections were deparaffinized and then treated with a citrate buffer (pH 6.0) in a microwave oven for 10–15 min to expose the antigens. Then, the sections were treated with 3% H_2O_2 to deactivate endogenous peroxidase. After blocking non-specific antigen binding with 5% BSA at 37 °C for 1 h, the sections were incubated with a specific primary antibody against VASN or HIF-1α (1:100 dilution, Abcam, USA) at 4 °C overnight. After incubating with the corresponding secondary antibodies at 37 °C for 1 h, the sections were stained with diaminobenzidine and counterstained with hematoxylin. Representative images were taken using a light microscope (Olympus, Japan).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The concentration and quality of RNA were analyzed by NanoDrop (Thermo, USA). Then, RNA was reverse-transcribed to cDNA by PrimeScript™RT kit (Takara, Japan). RT-qPCR assay was conducted by the SYBR Green PCR Master Mix Kit (Qiagen, Germany). Gene levels were determined using the $2^{-\Delta\Delta Ct}$ technique and adjusted to GAPDH.

Western blot

Total protein was extracted from cells using RIPA lysis buffer (Beyotime, China). The protein concentration was measured by the BCA Kit (Beyotime, China). The protein was loaded on 10% SDS-PAGE and transferred onto the PVDF membranes. Then, the membranes were blocked with 5% skim milk for 1 h. The membranes were incubated with the following primary antibodies overnight at 4˚C: anti-VASN (ab156868, 1: 1000 dilution, Abcam, USA), anti-N-Cadherin (ab76011, 1: 5000 dilution, Abcam, USA), anti-E-Cadherin (ab314063, 1: 1000 dilution, Abcam, USA), anti-YAP (#4912, 1: 1000 dilution, Cell Signaling Technology, USA), anti-TAZ (#4883, 1: 1000 dilution, Cell Signaling Technology, USA), anti-PTEN (#9552, 1: 1000 dilution, Cell Signaling Technology, USA), anti-P-AKT (ab38449, 1: 500 dilution, Abcam, USA), anti-AKT (#9272, 1: 1000 dilution, Cell Signaling Technology, USA), anti-HIF-1α (#3716, 1: 1000 dilution, Abcam, USA), anti-β-actin (ab6276, 1: 5000 dilution, Abcam, USA). After this, the membranes were incubated with the secondary antibody for 2 h. At last, the images were collected by ECL luminescence (Beyotime, China) and western blot imaging system. β-actin was used to normalize protein expression level.

Statistical analysis

Experimental results were independently repeated at least three times and presented as mean ± SD. The statistical analyses were performed using GraphPad Prism 6. A paired t-test was used to analyze the differences between two groups. One-way ANOVA followed Tukey's poc host was used to analyze the differences among multiple groups. $P < 0.05$ indicated a statistical significance.

Results

VASN is increased in high-grade bladder cancer tissues and cells

HE staining showed that non - tumor bladder tissues displayed a normal structure with well-arranged cell layers and intact muscularis propria (Fig. 1A). Besides, NMIBC tissues showed abnormal cell proliferation in the urothelium with loss of normal architecture but no muscle layer invasion, and MIBC tissues presented highly disorganized tumor cells invading the muscularis propria with evident nuclear atypia (Fig. 1A). Firstly, IHC was used to assess VASN expression in NMIBC tissues and MIBC tissues. The data showed that VASN was highly expressed in MIBC tissues relative to NMIBC tissues (Fig. 1B). Consistently, there also exhibited an elevated VASN mRNA and protein expression in bladder cancer cell lines (RT4 and T24) compared with ureteral epithelial cell line (SV-HUC-1) (Fig. 1C-D). It is worth noting that the levels of VASN mRNA and protein were markedly up-regulated in the high-grade bladder cancer cell line T24 compared to the low-grade bladder cancer cell line RT4 (Fig. 1C-D). These data support that VASN may play a role in the progression of bladder cancer.

Hypoxia promotes low-grade bladder cancer cell migration and EMT progression, and increases VASN expression

Recent studies have shown that hypoxia plays an important role in the metastasis of malignant tumors[17,24]. In order to investigate the changes in biological characteristics of poorly differentiated bladder cancer cells RT4 under hypoxia conditions, RT4 cells were cultured under normoxic conditions or hypoxic conditions for 48 h, and T24 cells were cultured under normoxic conditions. Wound healing and transwell assays showed that the migration ability of high-grade bladder cancer cell line T24 was significantly increased compared to the low-grade bladder cancer cell line RT4 (Fig. 2A-B). In addition, the migration number of RT4 cells under hypoxia was significantly higher than that in the normal oxygen concentration control group (Fig. 2A-B). Western bolt suggested that hypoxia significantly decreased E-Cadherin protein expression in RT4 cells, and increased N-Cadherin protein expression in RT4 cells (Fig. 2C). These data indicate that hypoxia induces the occurrence of migration and EMT in bladder cancer. Besides, the VASN level was up-regulated under hypoxia in RT4 cells (Fig. 2D). These data indicate that VASN participates in hypoxic adaptive response in bladder cancer.

Hypoxia-elevated VASN promotes migration of bladder cancer cells

To determine the function of VASN on bladder cancer, VASN knocked down or overexpressed to conduct lose- or gain-of-function assays. RT-qPCR assay showed after treatment with si-VASN, VASN expression was significantly decreased (Fig. 3A). Transfection of RT4 cells with VASN OE markedly increased VASN level (Fig. 3B). Functionally experiments showed that overexpression of VASN induced RT4 cell migration, while

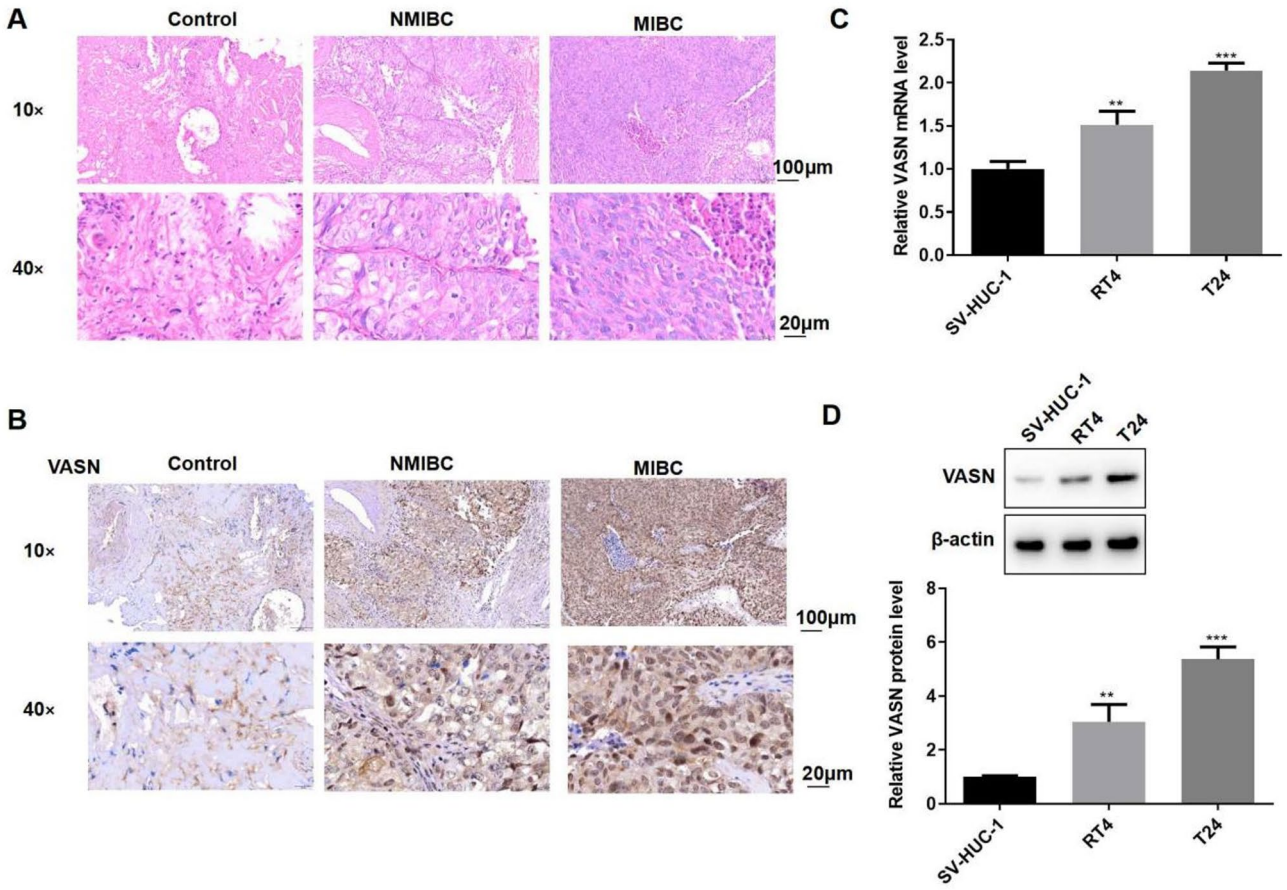

Fig. 1. VASN is increased in high-grade bladder cancer tissues and cells. (**A**) HE staining was used to observe the pathological morphology of bladder tissue in detail to reveal the changes of cell and tissue structure ($n = 3$). Scale bar, 100 μm; Scale bar, 20 μm. (**B**) IHC was used to assess VASN expression in NMIBC tissues and MIBC tissues ($n = 3$). Scale bar, 100 μm; Scale bar, 20 μm. (**C**) Relative mRNA expression level of VASN was examined using RT-qPCR in bladder cancer cell lines (RT4 and T24) and ureteral epithelial cell line (SV-HUC-1) ($n = 3$). (**D**) Relative protein expression level of VASN was examined using western blot in bladder cancer cell lines (RT4 and T24) and ureteral epithelial cell line (SV-HUC-1) ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. SV-HUC-1.

silencing VASN inhibited hypoxia-induced migration of RT4 cells (Fig. 3C-D). Collectively, these results indicate that hypoxia induces bladder cancer cell migration through up-regulating VASN.

VASN regulates the YAP/TAZ and PTEN/AKT pathways in bladder cancer cells

Recent studies have shown that the YAP/TAZ and PTEN/AKT pathways play an important role in hypoxia-induced tumor cell progression[25,26]. Therefore, we studied whether VASN would affect the YAP/TAZ and PTEN/AKT pathways in hypoxia-mediated RT4 cells. Western blot assay showed that the levels of VASN, YAP, TAZ and AKT phosphorylation were increased, and the level of PTEN was decreased in hypoxia-cultured RT4 cells compared with normal-cultured RT4 cells (Fig. 4). Besides, silencing VASN reversed the hypoxia-mediated changes in YAP/TAZ and PTEN/AKT pathways (Fig. 4). Collectively, these results suggest that the YAP/TAZ and PTEN/AKT pathways are involved in the promoting role of VASN on hypoxia-induced bladder cancer cell progression.

HIF-1α positively correlates with VASN

It's reported that HIF-1α is an important transcription factor that causes multiple gene up-regulation in response to decreased oxygen availability[27,28]. IHC showed that HIF-1α was increased in MIBC tissues compared to NMIBC tissues (Fig. 5A). The expression levels of HIF-1α and VASN were increased under hypoxia in a time-dependent manner (Fig. 5B). Spearman correlation analysis showed that VASN expression was positively correlated with HIF-1α expression (Fig. 5C). To explore the effect of HIF-1α on VASN expression, RT4 cells were knocked down HIF-1α. The transfection efficacy was verified by RT-qPCR. The expression of HIF-1α was decreased in RT4 cells with si-HIF-1α transfection (Fig. 5D). Besides, the knockdown of HIF-1α significantly decreased HIF-1α and VASN protein expression in hypoxia-cultured RT4 cells (Fig. 5E). These results suggest that HIF-1α activates hypoxia-induced VASN expression in bladder cancer cells.

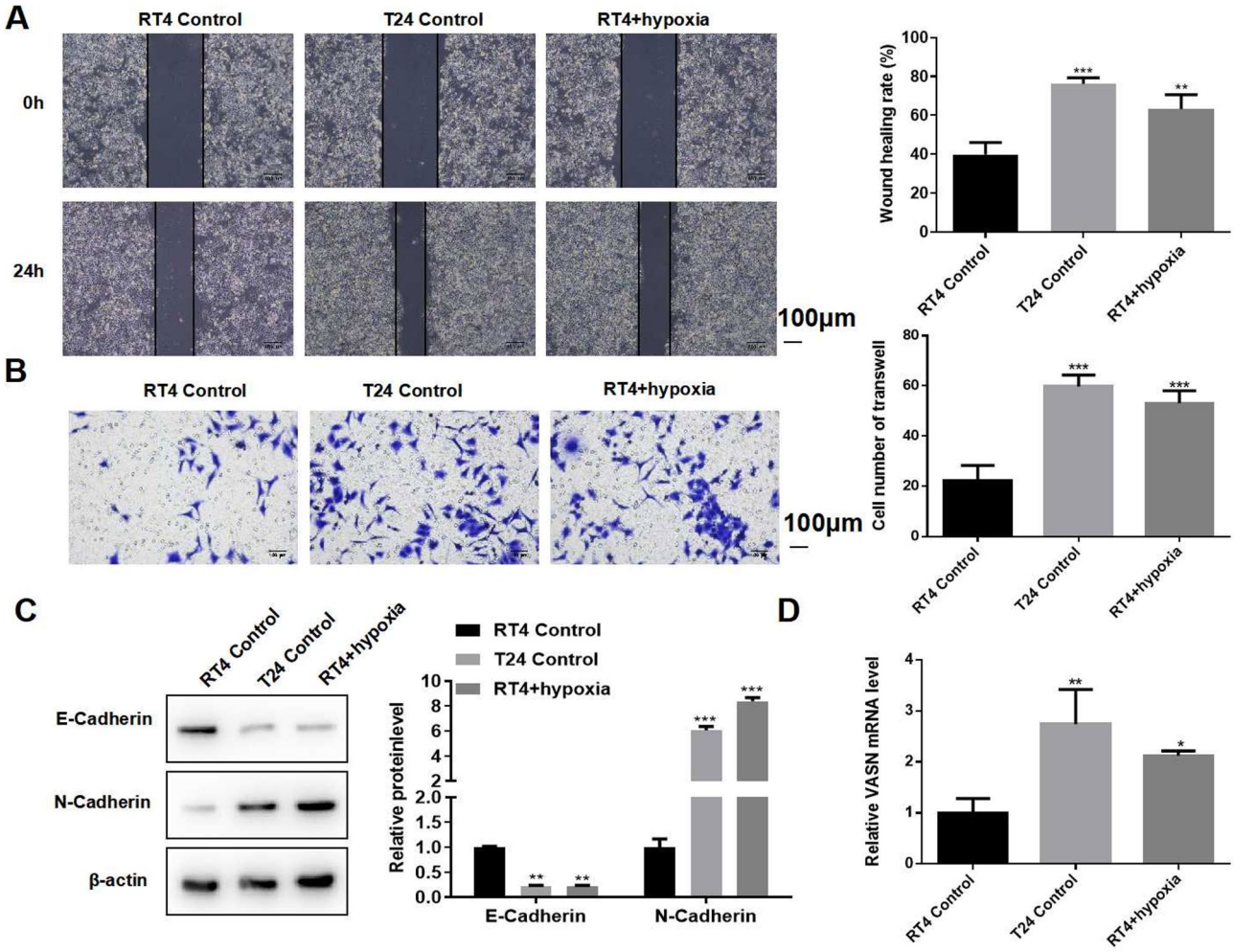

Fig. 2. Hypoxia promotes low-grade bladder cancer cell migration and EMT progression, and increases VASN expression. RT4 cells were cultured under normoxic or hypoxic conditions for 48 h, and T24 cells were cultured under normoxic conditions. (**A**) Wound healing was performed to investigate the migration ability of RT4 cells, T24 cells and hypoxia-mediated RT4 cells ($n = 3$). Scale bar, 100 μm. (**B**) Transwell assay was carried out to assess the migration ability of RT4 cells, T24 cells and hypoxia-mediated RT4 cells ($n = 3$). Scale bar, 100 μm. (**C**) Western blot was used to assess EMT-related proteins E-Cadherin and N-Cadherin expression ($n = 3$). (**D**) VASN mRNA level was detected by RT-qPCR ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RT4 Control.

Discussion

Hypoxia is an important feature of solid tumors, which activates related genes of tumor cells to adapt to the microenvironment[29]. According to the characteristics of lower tumor differentiation, higher malignancy and poor prognosis, hypoxia-induced dedifferentiation leads to tumor heterogeneity and malignant transformation of tumor cells, which increases the aggressiveness of tumor cells. This study aims to elucidate the biological significance of VASN in bladder cancer malignancy. In this study, the expression of VASN was detected in bladder cancer tissues and cells of different invasion grades. Furthermore, the hypoxic environment of bladder cancer was established in vitro to elucidate the biological characteristics of VASN in hypoxia-induced non-invasive bladder cancer cells. Our study showed that the migration and adhesion of bladder cancer cells were enhanced under hypoxia. Most importantly, to our knowledge, this is the first time that hypoxia up-regulation of VASN has been shown to promote bladder cancer growth and migration. This study provides a sufficient theoretical basis for further investigation into the role of the HIF-1α/VASN axis in bladder cancer metastasis.

Molecular targeted therapy is a new trend in cancer therapy[1,30]. VASN, a newly discovered protein molecule, is an important cell surface factor. VASN has been found to promote the proliferation and invasion of cancer cells, and induce the development process of cancer transformation[31,32]. A study suggested that VASN was highly expressed in lung adenocarcinoma, and overexpression of VASN significantly promoted the malignancy of lung adenocarcinoma, including accelerating tumor cell proliferation and invasion[31]. In addition, VASN has been reported to promote cell transformation and inhibit angiogenesis[33]. Chen W et al. found that Cigarette smoke extract (CSE) and benzo[a]pyrene diol epoxide (BPDE) induced the expression of VASN in human bronchial epithelial cells (HBECs). Down-regulation of VASN in HBECs significantly inhibited CSE-induced lung cancer

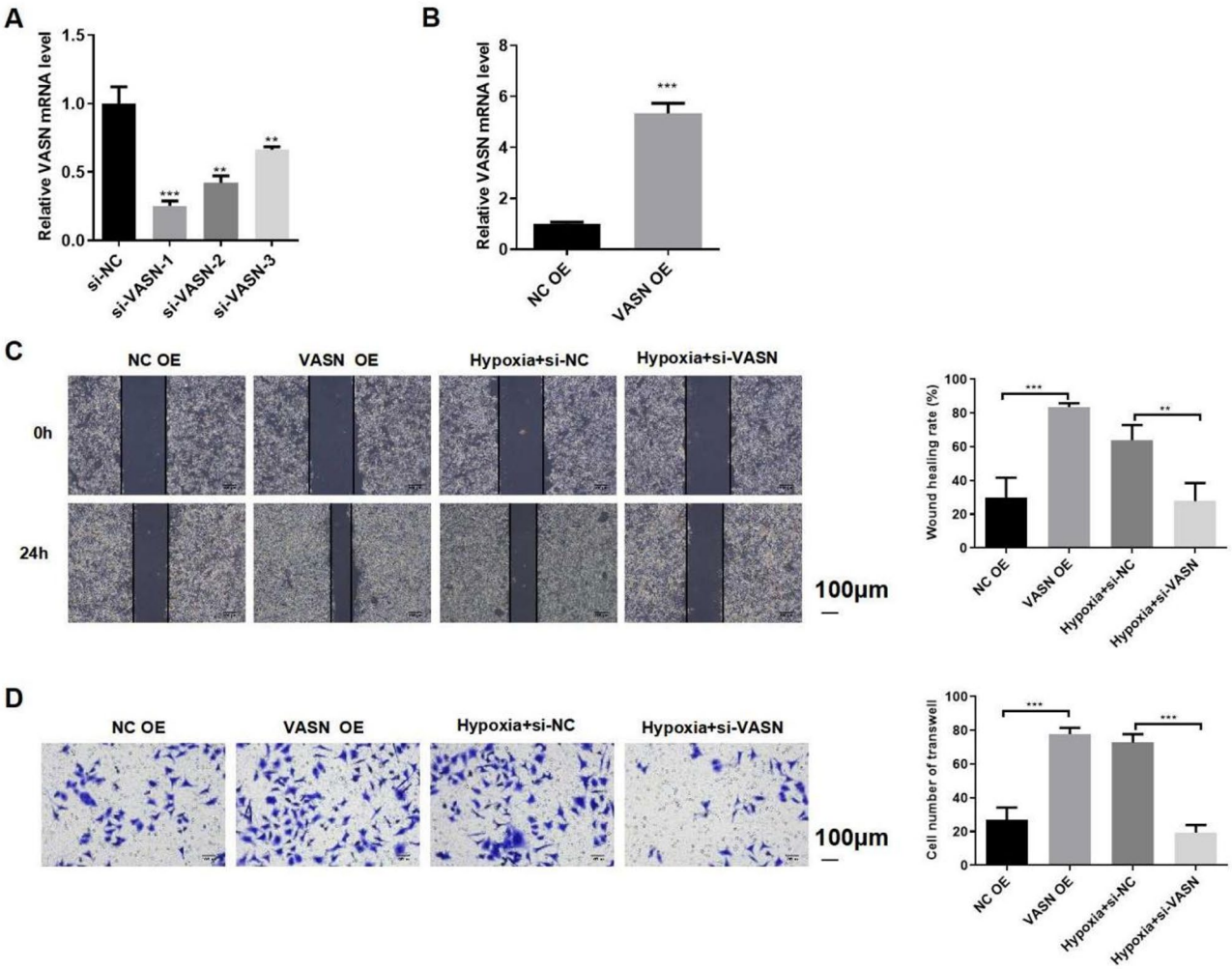

Fig. 3. Hypoxia-elevated VASN promotes migration of bladder cancer cells. (**A**) RT-qPCR assay was used to detect transfection efficiency of si-VASN-1, si-VASN-2, and si-VASN-3 ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. si-NC. (**B**) RT-qPCR assay was used to detect the transfection efficiency of VASN OE ($n = 3$). $^{***}P < 0.001$ vs. NC OE. RT4 cells were transfected with VASN OE, and hypoxia-mediated RT4 cells were transfected with si-VASN-1. (**C**) Wound healing was performed to investigate the migration ability of RT4 cells with corresponding treatment ($n = 3$). Scale bar, 100 μm. (**D**) Transwell assay was carried out to assess the migration ability of RT4 cells with corresponding treatment ($n = 3$). Scale bar, 100 μm. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. NC OE or Hypoxia + si-NC.

cell transformation[33]. VASN also participates in the immune inflammatory response[34]. In this study, we found that VASN was highly expressed in MIBC tissues relative to NMIBC tissues. Besides, VASN mRNA and protein expression levels were higher in bladder cancer cell lines (RT4 and T24) than ureteral epithelial cell line (SV-HUC-1). These data support that VASN may play a role in the progression of bladder cancer. This is consistent with a previous study in glioma[35]. A study reported that the expression of VASN was significantly correlated with the tumor grade of gliomas, and the expression was increased in high-grade gliomas[35].

It's reported that hypoxia in the tumor tissue promotes the occurrence of malignant phenotype and cell dedifferentiation, and also induces the change of downstream gene expression, thus inducing malignant transformation, heterogeneity, immune escape and treatment resistance of the tumor[29,36,37]. In the study, we also found that hypoxia promoted low-grade bladder cancer cell migration. Besides, hypoxia significantly decreased E-Cadherin protein expression, and increased N-Cadherin protein expression in RT4 cells, which promoted EMT progression. This is consistent with Lv WL's study[38]. A recent study showed that high expression of VASN in glioblastoma multiforme (GBM) cells protected cells from hypoxia-induced apoptosis[35]. Mechanistically, under hypoxia, the HIF-1α/STAT3 co-activating complex induced the expression of VASN in GSM[35]. A hypoxia-induced upregulation of VASN has been reported earlier in glioma stem like cells and also in a glaucoma model[39,40]. VASN, in turn seems to be able to modulate the HIF-1α/MAPK pathways in a mouse model of ischemia/reperfusion of the kidney[41]. However, the ole of VASN in hypoxic bladder cancer has not been reported in previous studies. Given that hypoxia is a characteristic feature of the tumor microenvironment in bladder cancer and is associated with poor prognosis, elucidating how VASN contributes to the cellular response to

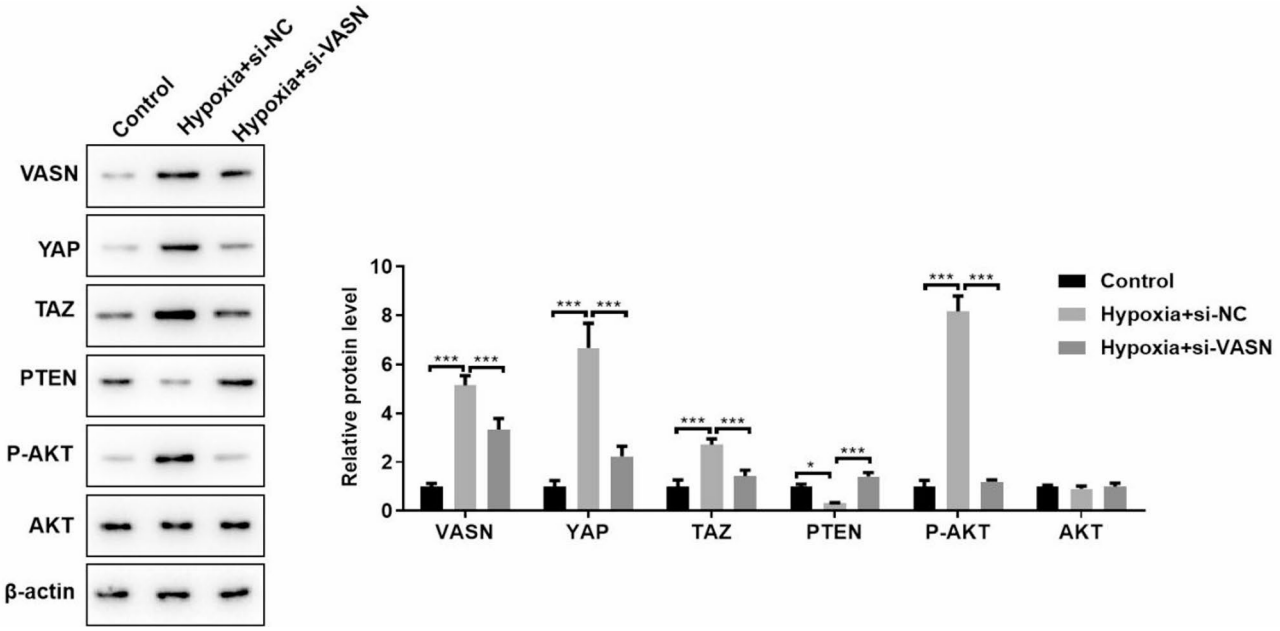

Fig. 4. VASN regulates the YAP/TAZ and PTEN/AKT pathways in bladder cancer cells. Western blot assay was used to assess YAP/TAZ and PTEN/AKT pathways ($n = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Control or Hypoxia + si-NC.

hypoxia may open up new avenues for developing targeted therapies. Our study revealed that hypoxia-induced bladder cancer cell migration through up-regulating VASN.

In addition, we also found that HIF-1α was increased in MIBC tissues compared to NMIBC tissues. Notably, VASN expression was positively correlated with HIF-1α expression. Regarding the rather untypical time course for HIF-1α protein stabilization with a maximum after 48 h, we speculated that the continuous hypoxic exposure for up to 48 h may lead to a cumulative effect on the stabilization of HIF-1α protein. As the cells are continuously exposed to a hypoxic environment, multiple cellular processes and signaling pathways are activated. Over time, these processes could interact and contribute to the increased stability of HIF-1α. Besides, this finding may also be related to the specific characteristics of the RT4 cells and the experimental conditions. This time course may differ from what has been reported in some other study[42]. However, it is important to note that the experimental models, cell types, and conditions used in those studies can vary significantly. To further validate and understand this finding, we plan to conduct additional experiments in the future. We will also perform more detailed molecular analyses to investigate the underlying mechanisms involved in the HIF-1α stabilization process, such as examining the expression and activity of key regulators of HIF-1α degradation. Our study further confirmed that HIF-1α activated VASN expression in hypoxia-induced bladder cancer cells. HIF-1α and its downstream pathway have been confirmed to be involved in the whole process of bladder cancer occurrence, invasion, metastasis, angiogenesis and drug resistance, which is closely related to the poor prognosis of patients[27,43,44].

YAP/TAZ pathway is essential in cancer initiation or growth in several cancers[45–47]. The activation of YAP/TAZ pathway induces cancer stem cell characterization, proliferation, chemotherapy resistance, and metastasis[46,47]. Gao Y et al. found that curcumin promoted KLF5 proteasome-dependent degradation by targeting YAP/TAZ in bladder cancer cells[48]. In bladder cancer cells, the activation of PTEN inhibited the expression of PI3K and AKT[49,50]. A large number of literatures have shown that the PTEN/AKT pathway is involved in regulating the proliferation, apoptosis and invasion of bladder cancer[49,50]. Recent studies have shown that the YAP/TAZ and PTEN/AKT pathways play an important role in hypoxia-induced tumor cell progression[25,26]. Our study revealed that the YAP/TAZ and PTEN/AKT pathways were involved in the promoting role of VASN on hypoxia-induced bladder cancer cell progression.

In this study, we provided valuable insights into the role of VASN in the context of bladder cancer, particularly in relation to the hypoxic microenvironment. We elucidated the role of VASN in the cellular response to hypoxia in bladder cancer cells, as well as its involvement in regulating relevant signaling pathways, which contributes to our understanding of the biological mechanisms underlying bladder cancer progression. However, it is essential to acknowledge several limitations of our study. One notable limitation is that there is currently a lack of clinical evidence directly linking VASN to the prognosis of bladder cancer patients. Our research primarily relied on in vitro experiments using bladder cancer cell lines, and while these findings provide valuable preliminary data, translating them to the clinical setting requires further investigation. To firmly establish a significant association between VASN and the prognosis of bladder cancer patients, large-scale, well-designed clinical studies involving a substantial number of patients are necessary. Such studies would involve analyzing the expression levels of VASN in bladder cancer tissues obtained from patients with known clinical outcomes, including overall survival,

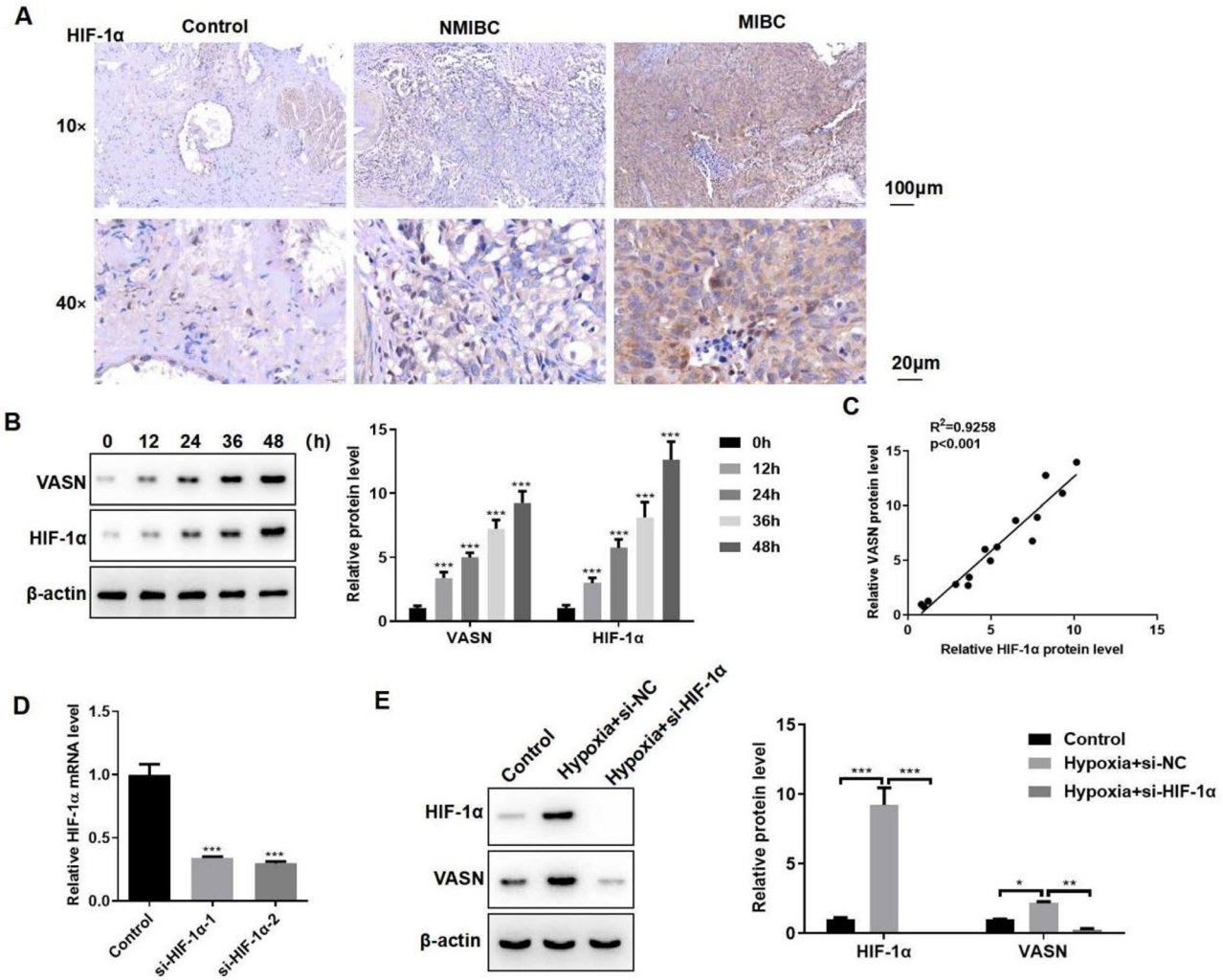

Fig. 5. HIF-1α positively correlates with VASN. (**A**) IHC was used to assess HIF-1α expression in NMIBC tissues and MIBC tissues ($n = 3$). Scale bar, 100 μm; Scale bar, 20 μm. (**B**) RT4 cells were incubated under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) at 37 °C for 12, 24, 36, and 48 h, then western blot was used to observe the time-dependent changes in HIF-1α and VASN expression ($n = 3$). (**C**) Spearman correlation analysis showed that VASN expression was positively correlated with HIF-1α expression ($n = 3$). (**D**) RT-qPCR assay was used to detect the transfection efficiency of si-HIF-1α-1 and si-HIF-1α-2 ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. si-NC. (**E**) Relative protein expression levels of VASN and HIF-1α were examined using western blot in RT4 cells with si-HIF-1α-2 transfection and under hypoxia for 48 h ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control or Hypoxia + si-NC.

disease-free survival, and recurrence rates. Building on the previous discussion about the limitations of our study regarding the significance of VASN in bladder cancer, another notable limitation that must be acknowledged is the absence of in vivo animal experiments. Additional in vivo studies are needed to confirm the clinical relevance of our results in preventing the migration and metastasis of bladder cancer. While our study has made important contributions to the understanding of VASN in bladder cancer, these limitations highlight the need for additional research in this area.

In summary, HIF-1α activated VASN expression in hypoxia-induced bladder cancer cells, and VASN promoted bladder cancer progression via the YAP/TAZ and PTEN/AKT pathways. These findings provided a new approach and strategy for the treatment of bladder cancer.

Data availability

Raw data related to the study are available from the corresponding author on reasonable request.

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Author contributions
Qian-jin Zhang, Chun-hui Liu, and Ke Wang wrote the main manuscript, Xue-yuan Mao performed the statistical analysis, specimen collection, and Yang Dong, Ming-yi Zang, and Wei Zhang prepared the images. Quansheng Yu and Lin Hao designed the experiments and provided financial support. All authors participated in the experiment.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical consideration
This study strictly adhered to the ethical principles outlined in the Declaration of Helsinki. The research protocol was reviewed and approved by the Medical Ethics Committee of Suqian First People's Hospital (Ethics Approval No.: 2023-SL-0054). Prior to study implementation, all participants provided written informed consent after being fully informed of the research objectives, procedures, and potential risks.

Additional information
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