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Original Article

Roles of VTCN1 in apoptosis and invasion in nasopharyngeal carcinoma

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Abstract: V-set domain containing T cell activation inhibitor 1 (VTCN1) also known as B7-H4 plays an important role in regulating T-cell activation, cytokine secretion and the development of cytotoxicity. However, the direct effect of VTCN1 on carcinoma was not studied clearly, especially on nasopharyngeal carcinoma (NPC). In this paper, we verified the potential effect and mechanism of VTCN1 on NPC. Cell proliferation was measured by the Cell Count Kit-8 (CCK-8). Cell cycle was assessed by flow cytometry as well as cell apoptosis. Real-time PCR and Western blot analysis were used to detect the expression of VTCN1, Bax, Bcl-2, caspase-3, MMP-2 and MMP-9 after cells were infected with VTCN1 shRNA interfering vector. The results revealed that low expression of VTCN1 could inhibit the proliferation and invasion of NPC cell lines and induce cell cycle arrest and apoptosis. The Bax, Bcl-2, caspase-3, MMP-2 and MMP-9 expression and phosphorylation of JAK2/STAT3 had a direct relationship with the reduction of VTCN1. Taken together, VTCN1 could affect the apoptosis and invasion of NPC, indicating that VTCN1 is a potential target for treating NPC.

Keywords: Nasopharyngeal carcinoma, VTCN1, apoptosis, invasion, JAK2/STAT3

Introduction

Nasopharyngeal carcinoma (NPC) is the most common head and neck cancer with the highest incidence observed in Southeast Asia and among the Chinese from southern China as well as Hong Kong [1]. Although aggressive concurrent chemoradiotherapy is the standard treatment modality for NPC at the locally advanced stage, approximately 30% to 40% of NPC patients fail with local recurrence and/or distant metastasis [2]. And survival time for NPC patients with metastatic or advanced disease is only 5 to 11 months [3]. Previous epidemiological studies suggest several etiological factors for NPC including genetic susceptibility, early-age exposure to chemical carcinogens and latent EBV infection [4]. Specific amplifications in NPC implicated several putative oncogenes in NPC, including Bcl-2, CCND1, MYC and PIK3CA, which show overexpression [5]. However, the precise genetic changes that are responsible for NPC progression are largely unknown. The development and progression of NPC may involve accumulation of multiple genetic alterations over a long period of time. In the present study, we focused on the expression of VTCN1 oncogene in NPC and examined its potential functions in NPC.

V-set domain containing T cell activation inhibitor 1 (VTCN1) is a B7 family member also known as B7-H4 plays important role in regulating T-cell activation, cytokine secretion and the development of cytotoxicity [6, 7]. VTCN1 has been implicated in cancers of the female reproductive system and investigated for its possible use as a biomarker for cancer, but there are no preclinical studies to demonstrate that VTCN1 is a molecular target for therapeutic intervention of cancer. VTCN1 inhibits the activation and proliferation of antigen-specific tumor-infiltrating CD^{8+} effector T cells, suggesting a profound translational value to VTCN1 in the context of anticancer immunotherapy [8]. Furthermore, the overexpression of VTCN1 correlates indeed with advanced disease stage and poor prognosis in cancer patients [9], as well as with increased tumorigenicity and invasiveness in cancer cells [10]. However, the mechanism of the direct effect on NPC is still not clear.

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a significant role in various physiological processes, including immune function, cell growth and differentiation [11, 12]. Recently, accumulating evidence indicates that abnormalities in the JAK/STAT pathway are involved in the oncogenesis of several cancers. Constitutive activation of STAT3 correlates with cell proliferation and inhibits apoptosis in pancreatic cancer [13], osteosarcoma [14] and gliomas [15]. Conversely, inhibition of JAK/STAT signaling suppresses cell growth and invasion and induces apoptosis in various cancers [16, 17]. More recently, STAT3 has also been found to be activated in NPC cases and plays a significant role in driving NPC progression and metastasis [18]. These published reports all demonstrate the crucial importance of the JAK/STAT pathway in tumorigenesis and progression.

Here, we report that VTCN1 is overexpressed in NPC cell lines at both mRNA and protein levels. Importantly, VTCN1 knockdown was also observed in a significant decrease in cell proliferation and invasion of NPC cell lines, accompanied with cell cycle arrest and apoptosis. We also report that VTCN1 knockdown lead to inactivation of JAK2/STAT3 signaling.

Material and methods

Cell culture

Human NPC cell lines CNE1, HONE-1, NP65 and HNE2 were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China) cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution in a 37°C incubator containing 5% CO_2.

Construction of stable cell lines

VTCN1 shRNA was obtained from Sangon Biotech Co., Ltd. (Shanghai, China) cloned into the lentiviral vector. Then recombinant lentivirus pLKO.1-EGFP-shRNA VTCN1 were collected 48 h after transfection of 239T cells by using lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacture's instruction, followed by infecting HNE2 cells. A scramble shRNA cloned into pLKO.1-EGFP was used as negative control (NC). Cells were analyzed 48 h after infection.

CCK-8 assay

Cells infected with VTCN1 shRNA were harvested and planted in 96-well plates. Then 10 μL cell counting assay kit-8 (CCK-8) solution (Dojindo Laboratories, Japan) was added to each well at 1 h before the endpoint of incubation and the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

Flow cytometry assay

For cell cycle assay, cells infected with VTCN1 shRNA were harvested, planted in 6-well plates and fixed in 70% ethanol at -20°C overnight. The cells were subsequently resuspended in staining solution, containing 20 μg/mL propidium iodide (PI, BD Biosciences, Franklin Lakes, NJ, USA) and 200 μg/mL RNase A (BD Biosciences) for 15 min in the dark at room temperature followed by flow cytometry analysis (BD Biosciences). For cell apoptosis assay, cells infected with VTCN1 shRNA were harvested, planted in 6-well plates and incubated with annexin V fluorescein isothiocyanate (FITC) and PI, prior to analysis by a flow cytometry.

Transwell assay

Cells infected with VTCN1 shRNA were harvested and seeded into the upper wells of precoated with Matrigel (BD Biosciences) transwell chamber. Cell culture medium, supplemented with 10% FBS, was added into the lower well of the chamber. After 48 h of incubation, the cells on the upper well and the membranes coated with Matrigel were swabbed with a Q-tip. The cells migrated into the lower well were fixed with methanol and stained with 0.5% methylrosanilnium chloride solution for 30 min. The cells were counted at a magnification of ×200 and the mean of cell numbers was recorded.

Real-time RT-PCR

Total RNAs were extracted from human NPC cell lines with TRIZOL reagent (Invitrogen Life Technologies) and stored at -80°C. Complementary DNA was synthesized with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA). Real-time PCR was performed using a standard SYBR Green PCR kit protocol on ABI7300 (Applied Biosystem, Shanghai, China) thermal cycler. GAPDH was used as internal controls for indicated genes. The ΔΔCt method

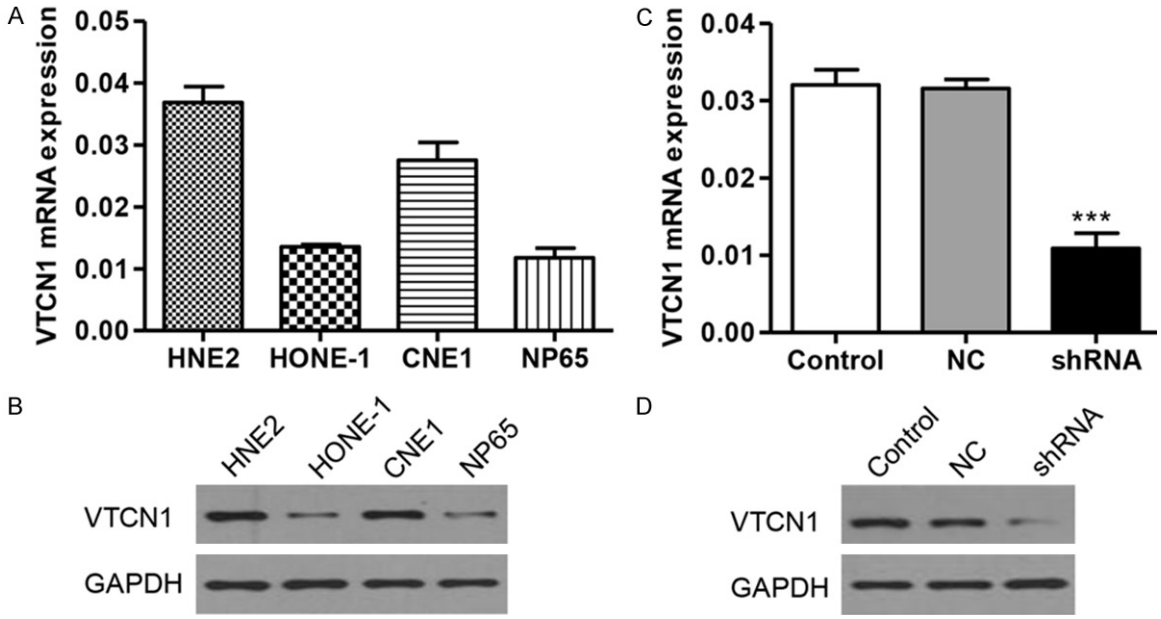

Figure 1. VTCN1 is up-regulated in NPC cell lines. A, B. The expression of VTCN1 in the four human NPC cell lines was assessed by Real-time PCR and Western blot. C, D. Successful knockdown of VTCN1 was confirmed by Real-time PCR and Western blot at 48 h after infection with VTCN1 shRNA lentivirus vector. ***P<0.001 compared with NC.

for relative quantification of gene expression was used to determine mRNA expression levels. The primers sequences (sense/antisense) used were as followed, VTCN1, 5'-AGGGAGTGGAGGAGGATACAG-3' (forward) and 5'-GCAGCAGCCAAAGAGACAG-3' (reverse); Bax, 5'-AGCTGAGCGAGTGTCTCAAG-3' (forward) and 5'-TGTCCAGCCCATGATGGTTC-3' (reverse); Bcl-2, 5'-AGACCGAAGTCCGCAGAACC-3' (forward) and 5'-GAGACCACACTGCCCTGTTG-3' (reverse); caspase-3, 5'-AACTGGACTGTGGCATTGAG-3' (forward) and 5'-ACAAAGCGACTGGATGAACC-3' (reverse); MMP-2, 5'-TTGACGGTAAGGACGGACTC-3' (forward) and 5'-GGCGTTCCCATACTTCACAC-3' (reverse); MMP-9, 5'-AAGGGCGTCGTGGTTCCAACTC-3' (forward) and 5'-AGCATTGCCGTCCTGGGTGTAG-3' (reverse); GAPDH, 5'-CACCCACTCCTCCACCTTTG-3' (forward) and 5'-CCACCACCCTGTTGCTGTAG-3' (reverse).

Protein extraction and Western blotting

Cells were harvested and lysed on ice for 30 min in RIPA buffer (Beyotime) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). Total protein extracts were separated on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Primary antibodies of VTCN1 (1:1000; Abcam), Bax (1:100; Santa Cruz), Bcl-2 (1:150; Santa Cruz), caspase-3 (1:1500; Abcam), MMP-2 (1:1000; Abcam), MMP-9 (1:500; Abcam), p-JAK2 (1:800; Cell Signaling Technology), JAK2 (1:1000; Cell Signaling Technology), p-STAT3 (1:1000; Abcam), STAT3 (1:1000; Abcam), and GAPDH (1:1500; Santa Cruz) were incubated at 4°C overnight. After washing with PBS 3 times, the membranes were incubated with their corresponding secondary antibodies at room temperature for 1 h. The membranes were washed again, and the antigen-antibody reaction was visualized by Amersham ECL detection system (GE Healthcare).

Statistical analysis

The data was presented as the mean value ± SD. Statistical significance was determined by the unpaired, two-tailed Student's t-test and One-way ANOVA analysis. SPSS 10.0 software was used to perform the statistical analyses. All the experiments were performed at least thrice in triplicates. P<0.05 was considered significant.

Results

VTCN1 expression in human NPC cell lines

The expression of VTCN1 was examined in four NPC cell lines (CNE1, HONE-1, NP65 and HNE2). Real-time PCR and Western blot analysis

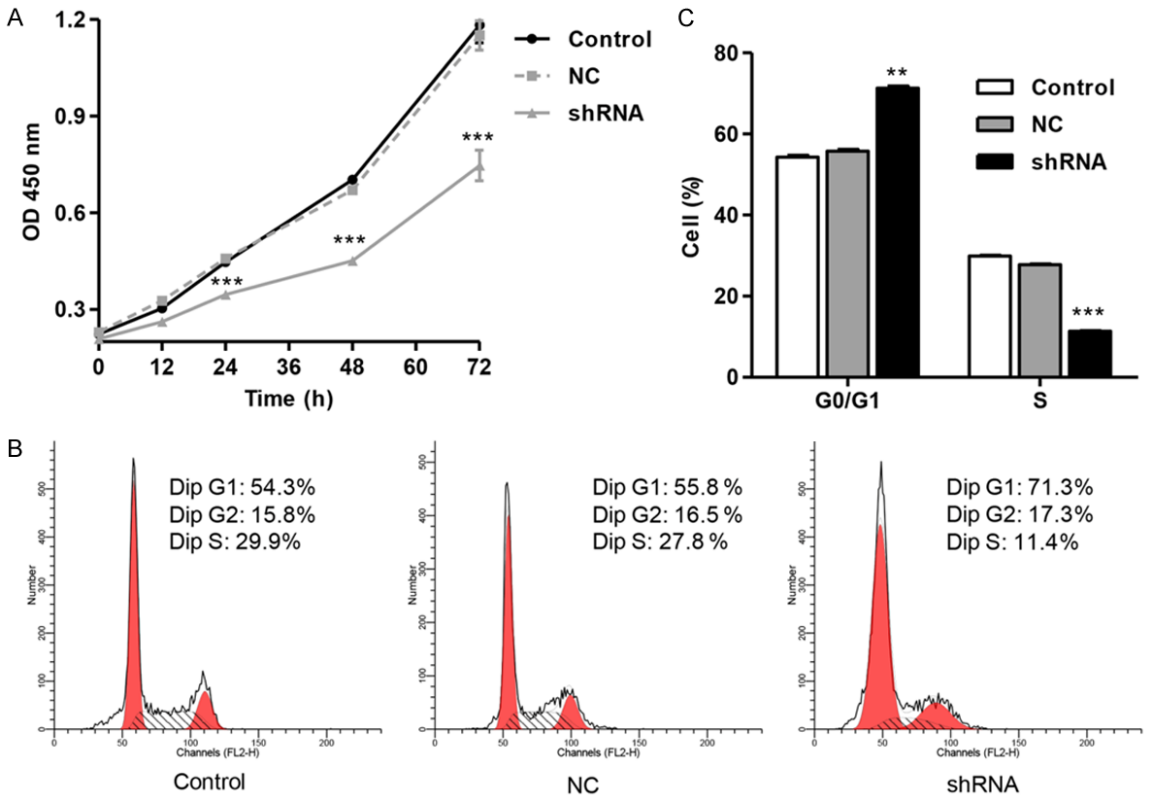

Figure 2. Effect of VTCN1 on NPC cell proliferation and cell cycle. A. HNE2 cells proliferation was detected using CCK-8 assay at 12, 24, 48 and 72 h. B, C. HNE2 cells cycle profile was analyzed using flow cytometry. ***P*<0.01 compared with NC, ****P*<0.001 compared with NC.

showed that higher level of VTCN1 mRNA expression was found in HNE2 cells compared with other cells, and the lower level of VTCN1 mRNA expression was found in HONE-1 and NP65 cells (**Figure 1A** and **1B**). Therefore HNE2 cell was therefore used for subsequent experiments.

To explore the biological significance of VTCN1 in NPC tumorigenesis, we established VTCN1 knockdown cell lines of HNE2 cells by stabilized pLKO.1-EGFP-shRNA VTCN1 infection. Expression of VTCN1 was confirmed by Real-time PCR and Western blot at 48 h after infection (**Figure 1C** and **1D**). The expression of VTCN1 was significantly decreased by 66.0% in the VTCN1 shRNA infected HNE2 cells. At the same time, the expression of VTCN1 remained at the same levels as in negative control HNE2 cells.

Effect of VTCN1 on cell proliferation and cell cycle of human NPC cell lines

48 h after infection, cell proliferation was analyzed through CCK-8 assay. CCK-8 assay revealed that VTCN1 shRNA significantly inhibited the cell proliferation in a time-dependent manner in HNE2 cells, decreased by 22.6%, 35.8% and 36.8% of that in NC group at 24, 48 and 72 h, respectively (**Figure 2A**).

Subsequently, the potential inhibitory effect of VTCN1 knockdown on cell cycle progression was investigated. As shown in **Figure 2B** and **2C**, the suppression of VTCN1 resulted in a higher number of cells in the G1 phase (mean=71.3) compared with NC controls (mean=55.8). There was a concomitant reduction in the number of cells in the S phase in HNE2 cells infected with VTCN1 shRNA.

Effect of VTCN1 on cell apoptosis and invasion of human NPC cell lines

To assess the effects of VTCN1 on cell apoptosis, annexin V/PI staining was performed. The apoptotic rate was significantly increased to the mean of 16.0% in HNE2 cells with VTCN1 shRNA infection compared with NC group (mean=4.7%; **Figure 3A** and **3B**). No significant

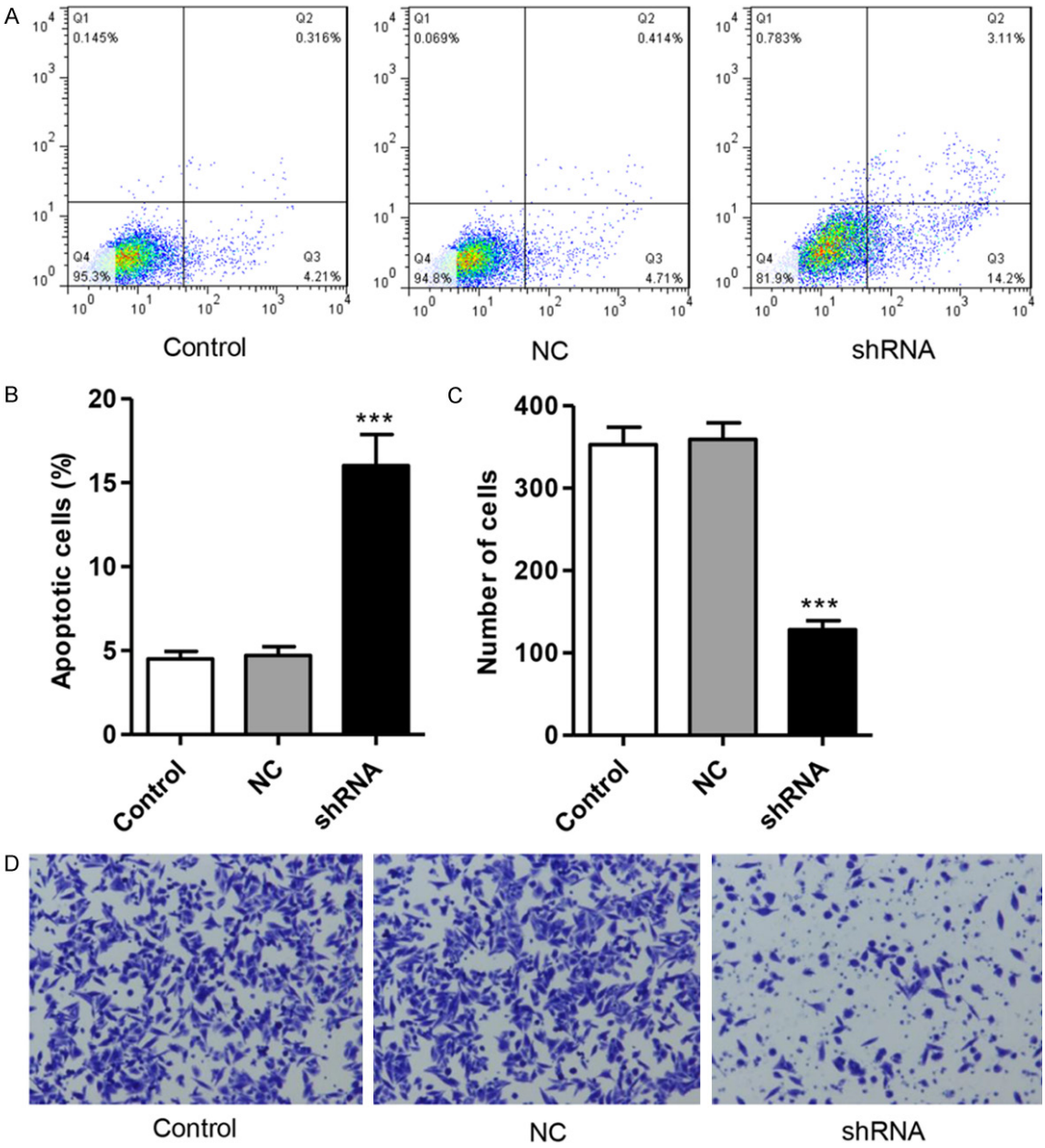

Figure 3. Effect of VTCN1 on NPC cell apoptosis and invasion. After infection with VTCN1 shRNA lentivirus vector. A, B. HNE2 cells were stained with annexin V-fluorescein and apoptotic rate was analyzed using flan cytometry. C, D. HNE2 cells invasion was performed by Transwell assay, and photographs were taken at 48 h after incubation in Matrigel precoated transwell chamber. ***P<0.001 compared with NC.

difference between the control and NC group was observed.

In the invasion assay, the invasive cells of HNE2 cells were significantly decreased with infection of VTCN1 shRNA 48 h after infection (mean=128; **Figure 3C** and **3D**), compared with the NC group (mean=359) at 48 h after infection. No significant difference between the control and NC group was observed.

Effect of VTCN1 on JAK2/STAT3 signaling and downstream proteins in NPC cell lines

To clarify the mechanism of VTCN1 implicated with cells apoptosis and invasion, the expression of the JAK2 and STAT3 phosphorylation were detected by Western blot. As shown in **Figure 4A**, VTCN1 knockdown significantly decreased JAK2 phosphorylation and STAT3 phosphorylation in HNE2 cells, while had no effect

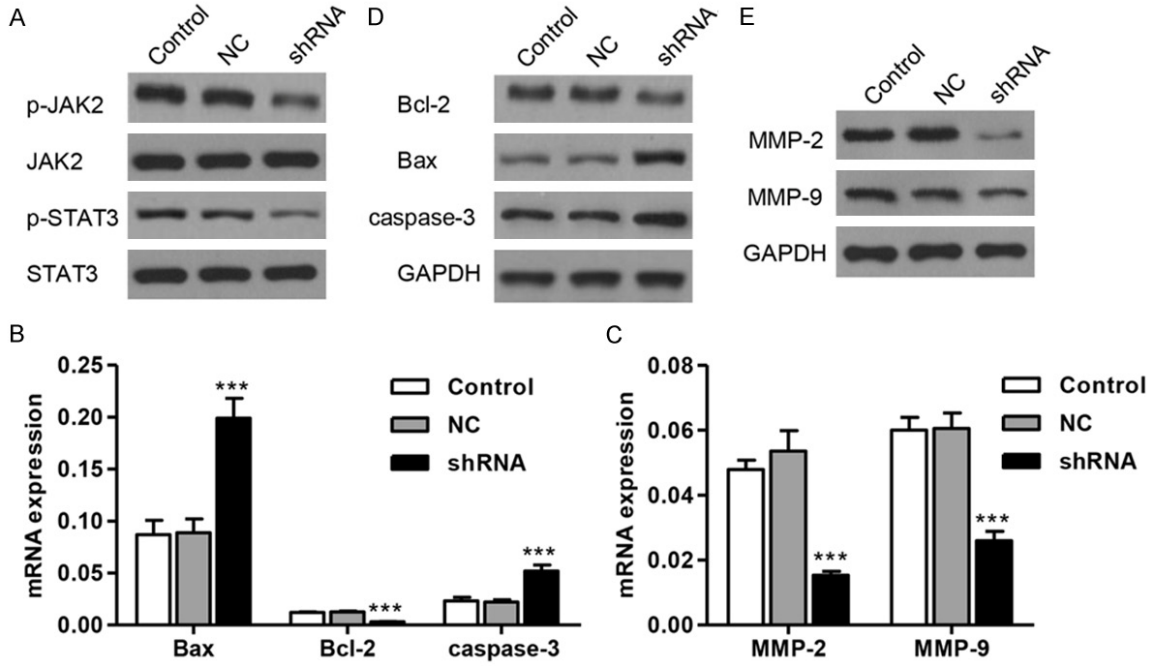

Figure 4. Effect of VTCN1 on JAK2/STAT3 phosphorylation and protein expressions in NPC cell lines. (A) After infection with VTCN1 shRNA lentivirus vector, JAK2 and STAT3 phosphorylation levels were measured in HNE2 cells by Western blot. The mRNA (B, C) and protein (D, E) levels of Bax, Bcl-2, Caspase-3, MMP-2 and MMP-9 were detected by Real-time PCR and Western blot in HNE2 cells. ***P<0.001 compared with NC.

on the protein levels of JAK2 and STAT3. These results suggest that down-regulation of VTCN1 suppresses activation of JAK2/STAT3 signaling in NPC cell lines.

Effect of VTCN1 on protein expressions in NPC cell lines

Then, three core proteins (Bax, Bcl-2 and caspase-3) in the apoptosis pathway and two core proteins (MMP-2 and MMP-9) in the invasion procession were predicted to be regulated by VTCN1. To experimentally validate VTCN1 regulation of these genes, we decreased expression of VTCN1 in HNE2 cells followed by detection of the expression levels of the proteins by Real-time PCR and Western blot. As shown in **Figure 4B** and **4C**, the mRNA levels of Bax and caspase-3 in HNE2 cells were remarkably increased by VTCN1 shRNA compared with NC group, with Bcl-2, MMP-2 and MMP-9 exception. The similar results were also found in HNE2 cells measured by Western blot (**Figure 4D** and **4E**).

Discussion

Knowledge about the precise molecular mechanisms underlying NPC tumorigenesis is crucial in the development of better therapeutic strategy for NPC patients. With the development of functional genomics, genes expression regulation was gradually extended from single gene linear regulation to multiple genes, especially to the oncogenes or tumor suppressor genes. In many prior papers, VTCN1 was proved to be a good predictable factor and target for many cancers. VTCN1 is not detectable in most tissues from healthy individuals but, *in vitro*, it is inducible in human immune cells [19]. Additionally, studies have reported the upregulation of VTCN1 in a variety of cancers including ovarian [9], breast [20] and lung cancer [21].

In the present study, we found that VTCN1 was up-regulated in NPC cell lines, with the highest expression detected in HNE2 cells and lowest expression of it in HONE-1 and NP65 cells, therefore, selected HNE2 cells to test the role of VTCN1 in the NPC tumorigenesis. The tumorigenic function of VTCN1 demonstrated by *in vitro* assays further supports its role as an oncogene in the development and progression of NPC. The results showed that knockdown of VTCN1 could inhibit cell proliferation and invasion and induce cell cycle arrest and apoptosis.

STAT3 is a member of a family of latent cytosolic transcription factors whose activation is by upstream kinases such as JAK2. Among the tumor-promoting activities ascribed to persistent STAT3 signaling are those involved with cell proliferation, metastasis, angiogenesis, and resistance to apoptosis [22]. Blockade of JAK2 and STAT3 activity decreases the expression of Bcl-2 and caspase-3 and increases Bax expression in gastric carcinoma [23], suggesting that the JAK2/STAT3 pathway may be involved in the regulation of the expression of Bax/Bcl-2 and caspase-3. The invasion-related MMP-2 and MMP-9 proteins were also positively correlated with STAT3 activation and could be decreased by AG490, a STAT3 pathway inhibitor, and increased by IL-6, a STAT3 pathway activator [24]. These results indicated that MMP-2 and MMP-9 may be the potential target proteins of STAT3.

To better understand the underlying mechanisms of VTCN1 induced NPC cell proliferation, apoptosis and invasion, we measured Bax, Bcl-2, caspase-3, MMP-2 and MMP-9 as regulatory protein of VTCN1 using Real-time PCR and Western blot. Pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins are known to regulate the apoptosis of NPC cells [25]. Bax plays a major role in the apoptotic response of NPC cells, the high expression of which is shown to correlate with an increased survival of NPC patients [26]. The Caspases, especially Caspase-3, are known to act downstream of Bax/Bcl-2 and play a key role in the execution of apoptosis [27]. In the present study, we found that knockdown of VTCN1 significantly increased expression of Bax/Bcl-2 and caspase-3 in HNE2 cells. MMPs, the gelatinases MMP-2 and MMP-9 in particular, have been implicated as contributing to the aggressiveness of highly metastatic NPC tumors [28, 29]. Our results suggest that VTCN1 may have a role in modulating the expression of MMP-2 and MMP-9 in HNE2 cells. It is likely that MMP-2 and MMP-9 expression in NPC is mainly involved in the degradation of extracellular matrix components for these tumors to invasion. However, as MMPs have been implicated in other processes during tumor progression, including angiogenesis [30], these enzymes may well be very important factors during NPC development.

In summary, at the cellular level, the present study demonstrated that knockdown of VTCN1 inhibits cell proliferation and invasion and induces cell cycle arrest and apoptosis. At the molecular level, p-JAK2 and p-STAT3 were inactivated in parallel with reduced anti-apoptotic protein (Bcl-2) and pro-invasion proteins (MMP-2 and MMP-9) and promoted pro-apoptotic proteins (Bax and caspase-3). In conclusion, our study indicated that VTCN1 may work as an oncogene and play a role in the treatment of NPC.

Disclosure of conflict of interest

None.

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