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PAG1 stimulates proliferation and metastasis of nasopharyngeal carcinoma through downregulating PTEN

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Abstract. – OBJECTIVE: We aim to uncover the expression pattern and biological functions of PAG1 in the progression of nasopharyngeal carcinoma (NPC).

PATIENTS AND METHODS: PAG1 levels in 28 paired NPC tissues and paracancerous tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Then, the potential influences of PAG1 on proliferative, migratory and invasive abilities of SUNE2 and CNE2 cells were assessed by cell counting kit-8 (CCK-8) and transwell assay, respectively. Next, the interaction between PAG1 and its direct target gene of phosphate and tension homology deleted on chromosome ten (PTEN) was verified by Dual-Luciferase reporter gene assay. At last, rescue experiments were conducted to uncover the role of PAG1/PTEN axis in the malignant progression of NPC.

RESULTS: PAG1 was highly expressed in NPC tissues and cell lines. Knockdown of PAG1 blocked NPC cells to proliferate, migrate, and invade. Dual-Luciferase reporter gene assay indicated the binding relationship between PAG1 and PTEN. In addition, both mRNA and protein levels of PTEN were negatively regulated by PAG1 in NPC cells. Notably, PTEN was responsible for PAG1-regulated malignant progression of NPC.

CONCLUSIONS: PAG1 is upregulated in NPC tissues and cells and stimulates the proliferative and metastatic abilities in NPC by targeting PTEN, thus aggravating the malignant progression of NPC.

Key Words:
PAG1, PTEN, Nasopharyngeal carcinoma (NPC), Metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is a highly prevalent malignancy in South China and Southeast Asia. According to the estimation proposed by the International Agency for Research on Cancer (IARC) in 2018, there were over 70,000 newly onset of NPC cases globally, and more than 80% of cases came from South China and Southeast Asia[1-3]. In China, over 97% of NPC cases belong to undifferentiated carcinoma (WHO III stage), which is highly sensitive to radiotherapy[4,5]. Clinical efficacy of early-stage NPC is up to 80-90%, and its 5-year survival is about 50%. Nevertheless, clinical outcomes of advanced NPC are extremely poor, with the 5-year survival of only 8-10%[6]. Multi-center clinical trials have uncovered that clinical stage is the determinant for clinical outcomes of NPC. It is reported that clinical efficacy of NPC decreases by 20% once the clinical stage increases one stage[7-9]. About 70% of NPC patients are confirmed in advanced stage (III-IV stage) at the initial diagnosis and their prognosis is relatively poor[10,11]. Therefore, it is necessary to uncover molecular mechanisms underlying the occurrence and metastasis of NPC, providing guidance for developing therapeutic strategies.

AG1 is a transmembrane adaptor protein that lacks protease activity, and acts as a scaffold during signal transduction, and provides the site for the integration of different pathways[12,13]. PAG1 is a Csk-binding protein (Cbp) consisting of 432 amino acids. It contains a short extracellular region with approximately 16-18 amino acids, a transmembrane region with 20 amino acids, and an intracytoplasmic region with 387-396 amino acids[14,15]. The cytoplasmic domain of PAG1 contains 10 tyrosine residues, 9 of which are potential substrates for Src kinase[16,17]. Among the Src-family tyrosine kinases, PAG1 can be phosphorylated by Fyn and Lck[17]. PAG1 extensively participates in biological processes, such as imprinting regulation, cell differentiation, and tumorigenesis[14,15].

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It is reported that PAG1 promotes the malignant progression of laryngeal carcinoma and breast cancer. Its biological role in NPC, however, remains unclear.

Gene of phosphate and tension homology deleted on chromosome ten (PTEN) is located on chromosome 10q23.3 and consists of 9 exons. PTEN encodes a protein consisting of 403 amino acids, and presents phosphatase activity[18,19]. PTEN is capable of blocking tumor development by antagonizing phosphorylase activities, such as tyrosine kinase[20,21]. Bioinformatics analyses have suggested that PAG1 can regulate the malignant progression of tumors by mediating PTEN level. Hence, it is believed that in-depth researches on PAG1 and PTEN in NPC progression may provide novel directions for developing diagnostic and therapeutic approaches. In this paper, PAG1 levels in 28 paired NPC and paracancerous tissues were tested. Regulatory effects of PAG1/PTEN axis on the malignant progression of NPC were further analyzed.

Patients and Methods

NPC Patients and Samples

NPC and paracancerous tissues were surgically resected from 28 NPC patients. All tumor tissues were pathological confirmed as squamous cell carcinoma. None of enrolled patients received preoperative anti-tumor therapy. Clinical data and follow-up data of enrolled patients were recorded. Tumor staging was conducted based on the guideline proposed by the Union for International Cancer Control (UICC). Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of The Second Children & Women's Healthcare of Jinan City and conducted in accordance with the Declaration of Helsinki.

Cell Culture

NPC cell lines (HNE1, SUNE2, HONE1, CNE2, and 6-10B) and human nasopharyngeal immortalized epithelial cell line (NP460) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) in a 5% CO_2 incubator at 37°C.

Transfection

Cells were inoculated in a 6-well plate and cultured to 40% confluence. Then, the cells were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 48 h and harvested for functional experiments. Finally, transfection plasmids (sh-NC and sh-PAG1) were constructed by GenePharma (Shanghai, China).

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Cell Migration and Invasion Assays

Cells were inoculated in a 24-well plate with 2.0×10^5/mL. 200 μL of suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, cells invading to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Next, invasive cell number was counted in 5 randomly selected fields per sample (magnification: 40×). Finally, migration assay was similarly conducted except for pre-coating of Matrigel in the bottom of transwell chambers.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. PAG1: Forward: 5'-GCAGCGGACAGATGCAGAT-3' and Reverse: 5'-CAGGAAGATGAGGAAGGTGATGA-3'; PTEN: Forward: 5'-CGACGGGAAGACAAGTTCAT-3' and Reverse: 5'-AGGTTTCCTCTGGTCCTGGT-3', and GAPDH: Forward: 5'-CCAACCGCGAGAAGATGA-3': Reverse: 5'-CCAGAGGCGTACAGGGATAG-3'.

Dual-Luciferase Reporter Gene Assay

CNE2 and SUNE2 cells inoculated in 24-well plates were co-transfected with WT-PAG1/MUT-PAG1 and pcDNA-PTEN/NC, respectively. 48 h later, cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

Western Blot

Cellular protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked in 5% skim milk for 1 h. The specific primary antibody was used to incubate with the membrane overnight at 4°C, followed by secondary antibody incubation for 2 h at room temperature. After Tris-Buffered Saline and Tween-20 (TBST) washing for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Kaplan-Meier method was introduced for survival analysis. Spearman correlation test was performed to assess the relationship between levels of PAG1 and PTEN with clinical parameters of NPC patients. $p<0.05$ was considered as statistically significant.

Results

PAG1 Was Upregulated in NPC Tissues

Compared with paracancerous tissues, PAG1 was upregulated in NPC tissues (Figure 1A). Consistently, PAG1 was highly expressed in NPC cell lines than that in human nasopharyngeal immortalized epithelial cell line (Figure 1B). In particular, CNE2 and SUNE2 cells expressed the highest level of PAG1 among the five tested NPC cell lines, which were selected in the following experiments.

PAG1 Expression Was Correlated With Lymphatic Metastasis, Distant Metastasis and Overall Survival in NPC Patients

Based on the median level of PAG1 in the enrolled 28 NPC patients, these patients were assigned into high-level and low-level group, respectively. Chi-square test was applied for analyzing the correlation between PAG1 level and clinical parameters of NPC patients. The data illustrated that PAG1 level was positively correlated with lymphatic metastasis and distant metastasis, rather than age, sex, and clinical stage of NPC patients (Table I). Meanwhile, Kaplan-Meier curves showed worse prognosis in NPC patients expressing a high level of PAG1 ($p<0.05$) (Figure 1C).

Knockdown of PAG1 Blocked NPC to Proliferate and Metastasize

To uncover the biological function of PAG1 in NPC progression, transfection efficacy of sh-

Table I. Association of PAG1 and PTEN expression with clinicopathologic characteristics of nasopharyngeal carcinoma.

Parameters	No. of cases	PAG1 expression		*p*-value	PTEN expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.077			0.305
<60	9	7	2		2	7	
≥60	19	8	11		8	11	
Gender				0.256			0.430
Male	14	9	5		4	10	
Female	14	6	8		6	8	
T stage				0.743			0.820
T1-T2	16	9	7		6	10	
T3-T4	12	6	6		4	8	
Lymph node metastasis				0.022			0.010
No	19	13	6		4	15	
Yes	9	2	7		6	3	
Distant metastasis				0.006			0.006
No	20	14	6		4	16	
Yes	8	1	7		6	2	

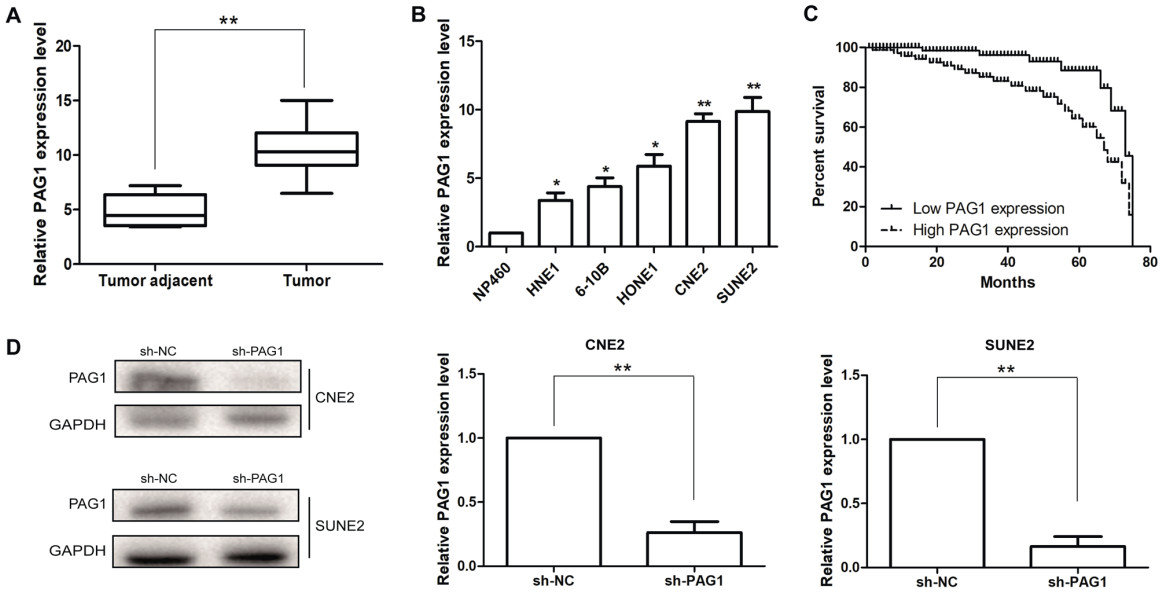

Figure 1. PAG1 is upregulated in NPC tissues and cell lines. **A,** PAG1 levels in NPC tissues and paracancerous tissues. **B,** PAG1 level in NPC cell lines and human nasopharyngeal immortalized epithelial cell line. **C,** Overall survival in NPC patients expressing high or low level of PAG1. **D,** Protein and mRNA levels of PAG1 in CNE2 and SUNE2 cells transfected with sh-NC or sh-PAG1. Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$.

PAG1 was firstly verified. Transfection of sh-PAG1 markedly downregulated protein and mRNA levels of PAG1 in both CNE2 and SUNE2 cells (Figure 1D). CCK-8 results depicted decreased viability in NPC cells with PAG1 knockdown (Figure 2A). Similarly, migratory and invasive abilities were attenuated after transfection of sh-PAG1 in CNE2 and SUNE2 cells (Figure 2B).

PTEN Was a Direct Target of PAG1

Dual-Luciferase reporter gene assay was conducted to validate the binding between PTEN and PAG1. It was shown that overexpression of PTEN markedly quenched Luciferase activity in wild-type PAG1 vector, rather than that in mutant-type one (Figure 3A). In the meantime, protein and mRNA levels of PTEN were upregulated after transfection of sh-PAG1 in NPC cells (Figure 3B, 3C). Compared with that in paracancerous tissues, PTEN was downregulated in NPC tissues (Figure 3D). Besides, worse prognosis was observed in NPC patients expressing a low level of PTEN (Figure 3E). In addition, the analysis of this study found that PTEN level was negatively correlated with lymphatic and distant metastasis of NPC patients, while it was unrelated to other clinical parameters (Table I).

PAG1/PTEN Axis Was Responsible for Regulating the Progression of NPC

The above data proved the interaction between PAG1 and PTEN. Thereafter, the involvement of PTEN in PAG1-influenced malignant progression of NPC was speculated. Knockdown of PAG1 could reverse the upregulated level of PAG1 in NPC cells transfected with si-PTEN (Figure 4A). Of note, silence of PTEN markedly elevated cell viability in NPC, which was abolished by knockdown of PAG1 (Figure 4B). The promotive effects of downregulated PTEN on migratory and invasive abilities in NPC were abolished by PAG1 knockdown (Figure 4C). Hence, it is verified that PAG1 promotes proliferative and metastatic abilities in NPC by downregulating PTEN.

Discussion

NPC is a malignant cancer originating from head and neck, which is characterized by evident race and geographic distribution. Insidious onset location, atypical symptoms, high malignant level, and high metastatic rate of NPC result in its poor prognosis[1-3]. Most of NPC patients develop cervical lymphatic metastasis or distant metastasis at the initial diagnosis[4,5].

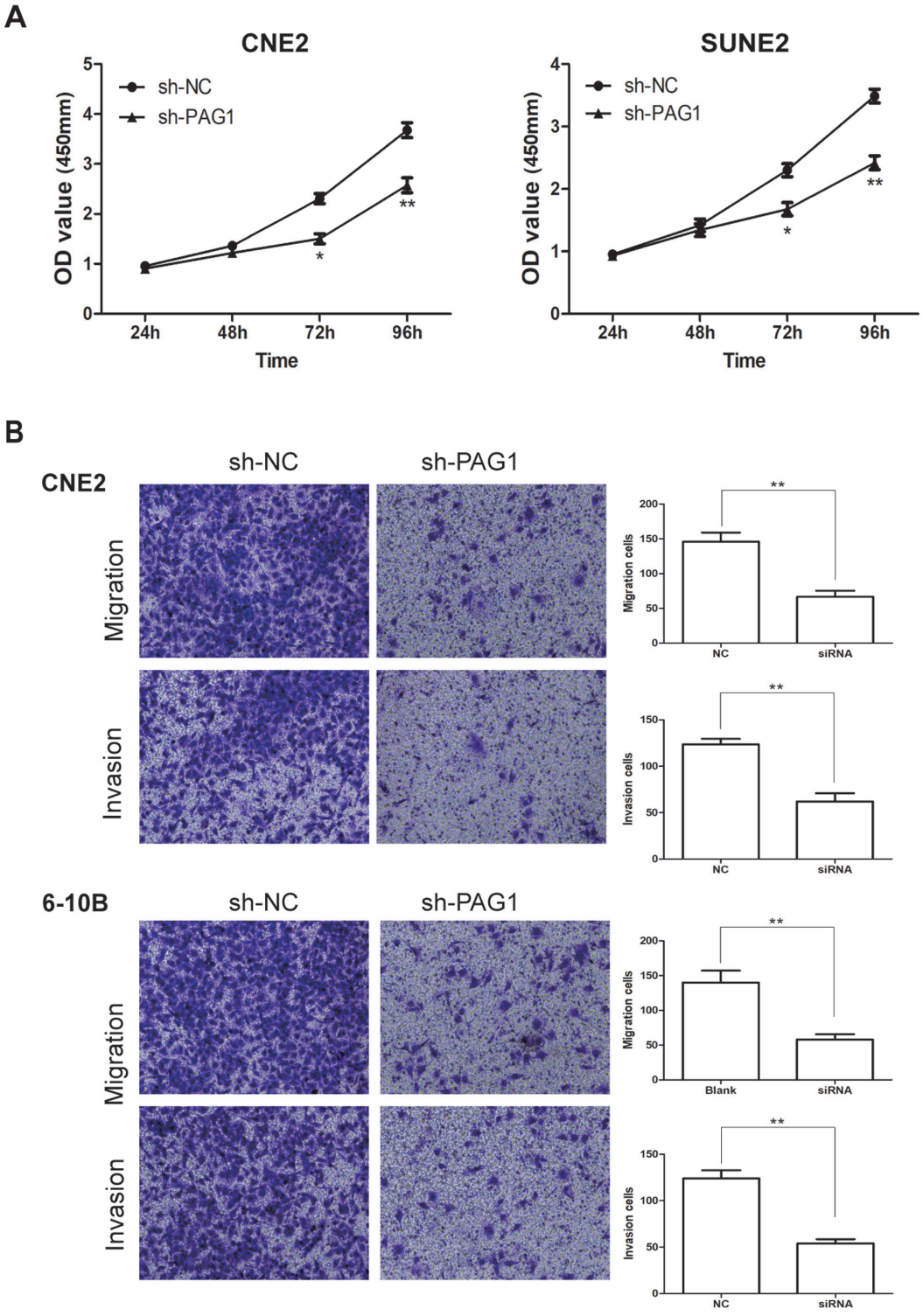

Figure 2. Knockdown of PAG1 blocks NPC to proliferate and metastasize. **A,** CCK-8 assay reveals viability in CNE2 and SUNE2 cells transfected with sh-NC or sh-PAG1. **B,** Transwell assay reveals migration and invasion in CNE2 and SUNE2 cells transfected with sh-NC or sh-PAG1 (magnification: 40×). Data were expressed as mean ± SD. *p<0.05, **p<0.01.

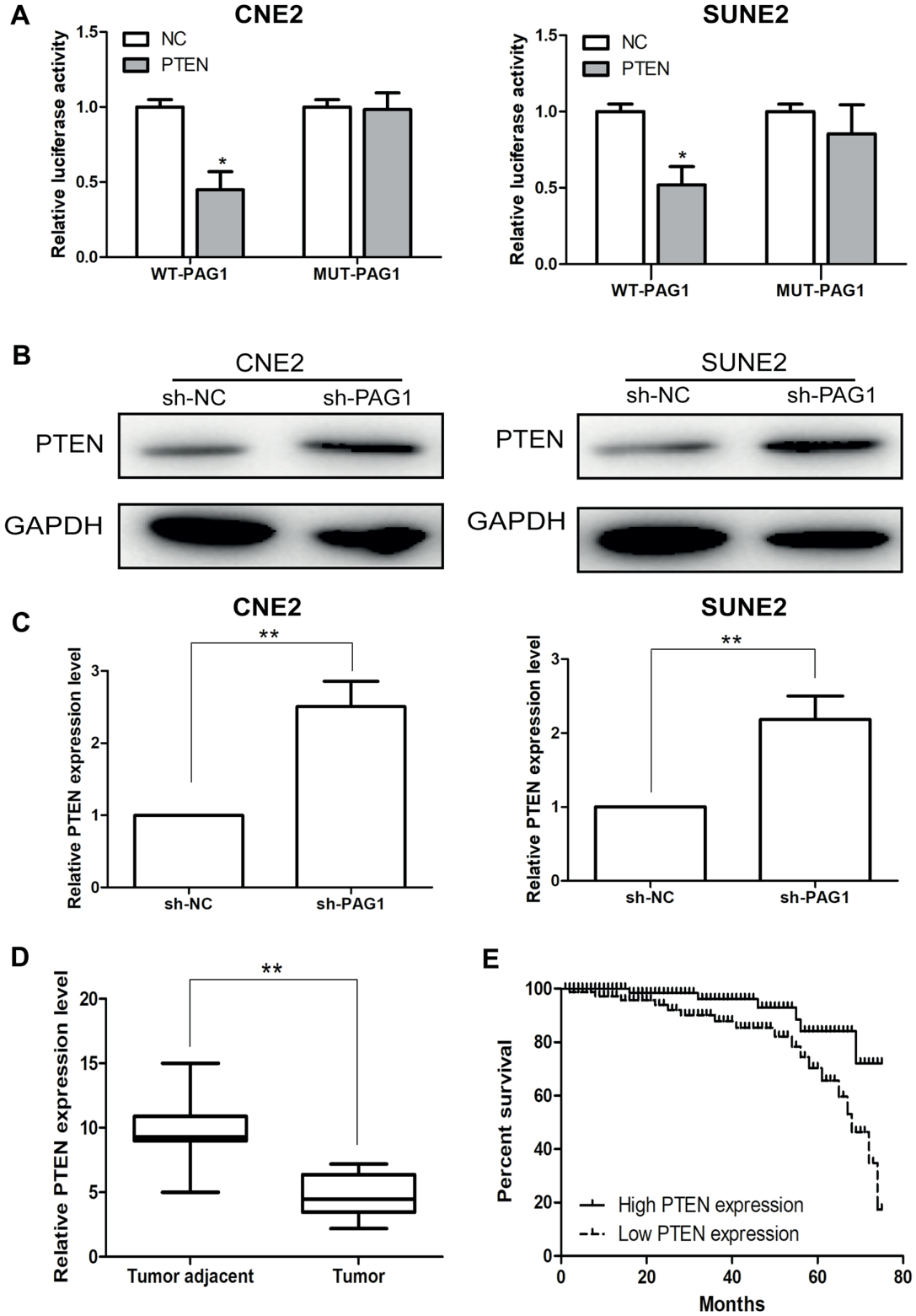

Figure 3. PTEN is a direct target of PAG1. **A,** Luciferase activity in CNE2 and SUNE2 cells co-transfected with WT-PAG1/MUT-PAG1 and pcDNA-PTEN/NC, respectively. **B,** Protein level of PTEN in CNE2 and SUNE2 cells transfected with sh-NC or sh-PAG1. **C,** The mRNA level of PTEN in CNE2 and SUNE2 cells transfected with sh-NC or sh-PAG1. **D,** PTEN levels in NPC tissues and paracancerous tissues. **E,** Overall survival in NPC patients expressing a high or low level of PTEN. Data are expressed as mean ± SD. *p<0.05, **p<0.01.

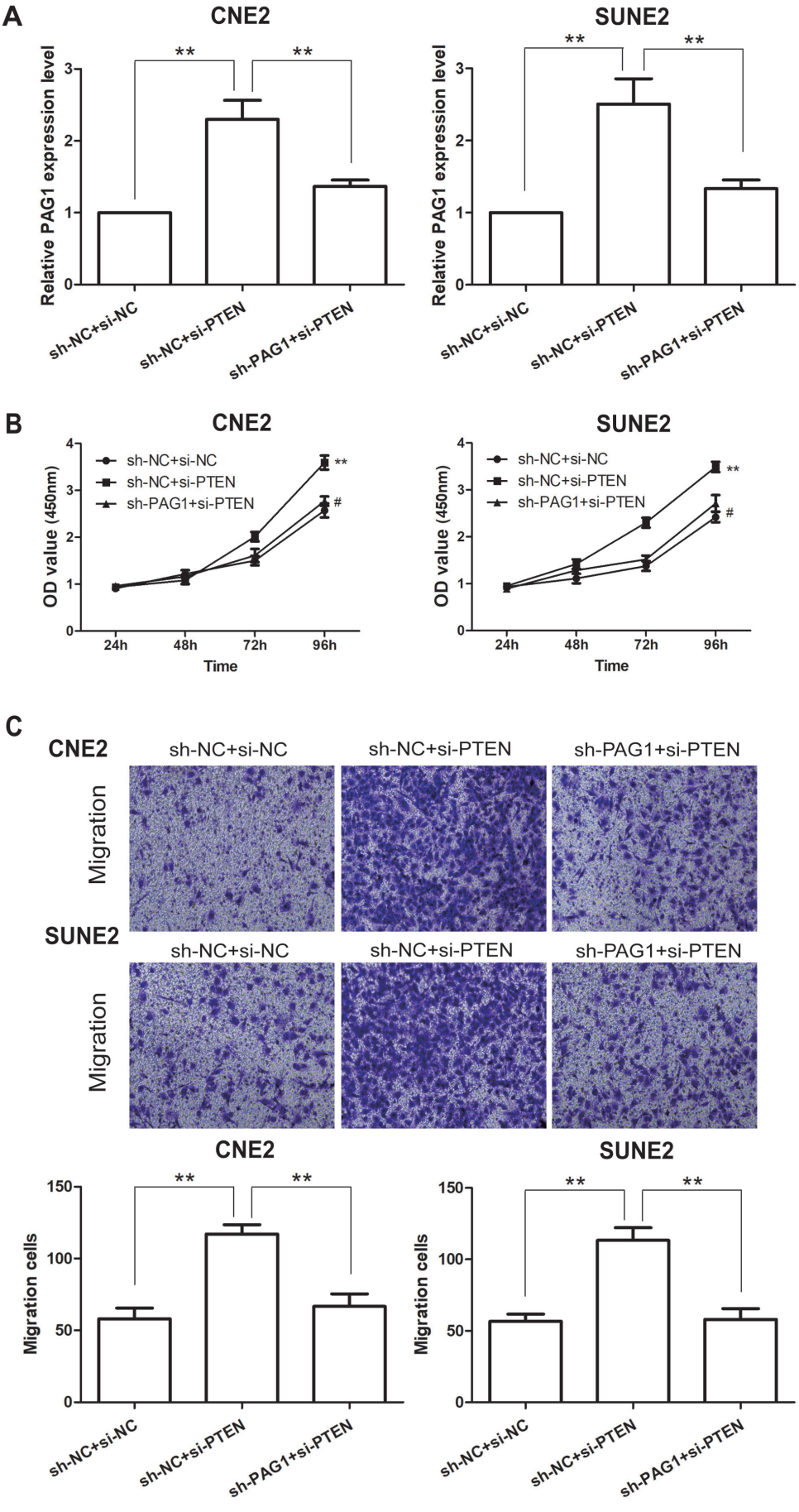

Figure 4. PAG1/PTEN axis is responsible for regulating the progression of NPC. CNE2 and SUNE2 cells were transfected with sh-NC + si-NC, sh-NC + si-PTEN or sh-PAG1 + si-PTEN. **A,** Relative level of PAG1. **B,** Cell viability. **C,** Migration and invasion (magnification: 40×). Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$.

Currently, radiotherapy combined with chemotherapy markedly improves the control rate of local NPC. The overall survival of NPC, so far, remains at 70%[6-9]. Further researches[20,21] are required to clarify molecular mechanisms of NPC.

Transcription changes of various genes are closely linked to the occurrence and progression of tumors. Altered transcriptional information results in changes in chromatin remodeling, splicing, and regulation of transcription factors, cell cycle progression, and cell apoptosis, thus leading to tumorigenesis[22,23]. During the process, the balance between oncogenes and tumor-suppressor genes is broken[24]. PAG1 is initially considered as a critical regulator of T cell activation. As a tyrosine phosphorylation protein, PAG1 binds to the SI-12 domain of the tyrosine kinase CSK, the major negative regulator of Src kinase, thereafter, resulting in excessive aggregation of CSK. Eventually, kinases in the Src family are suppressed through a negative feedback, and thus resting T cells remain dormant[12-17]. Shen et al[14] and Lu et al[15] have demonstrated the close involvement of PAG1 in tumor development. Therefore, our aim was firstly to elucidate the oncogenic role of PAG1 in the progression of NPC, as well as the specific mechanism of PAG1 regulating PTEN. The results revealed that PAG1 was upregulated in NPC tissues and cells. *In vitro* experiments verified the promotive effects of PAG1 on proliferative and metastatic abilities in NPC cells.

PTEN mutation has been identified in many types of tumors, which is a popular tumor-suppressor gene after the discovery of p53[18,19]. PTEN is inactivated mainly by allelic deletion, gene mutation, and methylation[19]. Previous studies[20,21] have uncovered the involvement of PTEN in tumor progression. Through bioinformatics prediction, it was found that PAG1 could bind to PTEN mRNA 3'UTR through seed sequences. Subsequently, the direct binding and regulatory relationship between PAG1 and PTEN were verified through Dual-Luciferase reporter gene assay, qRT-PCR, and Western blot. Notably, silence of PAG1 could partially reverse regulatory effects of PTEN on proliferative and metastatic abilities in NPC cells. Collectively, a negative feedback loop PAG1/PTEN has been confirmed to aggravate the malignant progression of NPC.

Conclusions

The results of this study demonstrated that PAG1 is upregulated in NPC tissues and cells and stimulates the proliferative and metastatic abilities in NPC by targeting PTEN, thus aggravating the malignant progression of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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