

Opa interacting protein 5 promotes metastasis of nasopharyngeal carcinoma cells by promoting EMT via modulation of JAK2/STAT3 signal

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Abstract. – **OBJECTIVE:** Opa interacting protein 5 (OIP5), as a tumor promoter gene, has emerged as a regulator in several types of tumors. However, the role of OIP5 in nasopharyngeal carcinoma (NPC) has not been reported. In this study, we aimed to explore the expression and biological function of OIP5 in NPC.

PATIENTS AND METHODS: The lung cancer datasets GSE12452 and GSE53819 were downloaded from the Gene Expression Omnibus (GEO) repository. Real-time-Polymerase Chain Reaction (RT-PCR) was performed to detect the expression levels of OIP5 mRNA. Cell Counting Kit-8 (CCK-8), colony-formation assay, wound healing assay and transwell assay were conducted to measure cells' proliferation, migration and invasion. Flow cytometry was used for analysis of apoptosis. Western blot assays were used to assess the effects of OIP5 on EMT and JAK2/STAT3 pathway.

RESULTS: The up-regulation of OIP5 mRNA was observed in NPC tissues from both GSE12452 and GSE53819. The results of RT-PCR also showed that the expression of OIP5 mRNA was significantly up-regulated in several NPC cell lines compared to normal nasopharyngeal cells. Furthermore, lost-function assay revealed that the knockdown of OIP5 markedly suppressed NPC cells proliferation, migration and invasion, and promoted cell apoptosis. In addition, the results of Western blot showed that silencing of OIP5 suppressed the EMT in NPC cell line. Meanwhile, the knockdown of OIP5 remarkably decreased the expression of p-JAK2 and p-STAT3 protein in both CNE1 and SUNE1 cells.

CONCLUSIONS: Our data indicated that OIP5 was highly expressed in NPC and promoted NPC progression by modulating JAK2/STAT3; our results shed light on utilizing OIP5 as a potential novel therapeutic target for the treatment of NPC.

Key Words:

OIP5, Nasopharyngeal carcinoma, JAK2/STAT3, EMT, Metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous, squamous cell malignancy arising from the epithelial cells that line the nasopharynx and causes 80,000 new cases and 50,000 deaths every year^{1,2}. It has been confirmed that three major etiologic factors, including genetic susceptibility, endemic environmental factors and Epstein-Barr virus (EBV) infection, are considered to induce NPC^{3,4}. Despite considerable advances in multimodal treatment, the 5-year survival rate of most middle-late- and late-stage NPC patients remains poor^{5,6}. Local recurrence and distant metastasis are the major cause of treatment failure and NPC related deaths⁷. In addition, drug resistance may hamper the efficacy of anti-cancer drugs⁸. Thus, the identification of promising therapeutic targets for patients with advanced disease is urgently needed. Opa interacting protein 5 (OIP5), also called LINT-2511 and hMis18beta, belongs to cancer/testis antigens (CTAs), which is necessary for centromere/kinetochore structure and biological function^{9,10}. This gene plays an important regulatory role in cell cycle progression *via* the E2F-Rb pathway. Evidence shows that this function was mediated by interacting with the retinoblastoma protein and lamina-associated polypeptide2 α ^{11,12}. Overall, these findings highlight the critical role of OIP5 in the cellular procedure. Of note, the

dysregulation of OIP5 was frequently reported in several tumors, such as bladder cancer¹³, glioblastoma¹⁴, gastric cancer¹⁵ and breast cancer¹⁶; the up-regulation of OIP5 revealed its oncogenic role in tumors, which has been demonstrated by *in vitro* and *in vivo*. On the other hand, the various molecular mechanism of OIP5 in tumors cells progression was uncovered^{17,18}. However, to our best knowledge, whether OIP5 was abnormally expressed in NPC remains largely unclear, and its biological function in NPC has not been investigated. In this work, we explored the expression pattern of OIP5 in NPC by analyzing GEO datasets, confirming that OIP5 was highly expressed in NPC tissues. Then, *via* detecting the expression levels of OIP5 in NPC cells, we found that the expression of OIP5 was also up-regulated in NPC. These results, together with the previous study, indicated that OIP5 was an oncogenic gene in NPC progression. Subsequently, we performed a lost-function assay to further demonstrate the potential function of OIP5. Mechanistically, OIP5 may display its oncogenic effect in the activation of JAK2-STAT3 signaling, a well-studied tumor-related signaling pathway which was frequently activated in various tumor progression. Our data indicated that OIP5 could be a potential therapeutic target for the treatment of NPC patients.

Patients and Methods

Cell Lines and Cell Transfection

The human immortalized nasopharyngeal epithelial cell line NP69 was purchased from the FengHui Biotechnology Co., Ltd. (Changsha, Hunan, China). The human NPC cell lines (SUNE-1, CNE-2, HONE-1, CNE-1, HNE-1 and C666-1) were purchased from the Chinese Academy of Sciences Cell Bank (Xuhui, Shanghai, China). The cells were all grown in Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY,

USA) as well as 1% penicillin-streptomycin mix solution (YouKang, Haidian, Beijing, China) with 5% CO₂ at 37°C. The study was approved by the Ethics Committee of Inner People's Hospital and Inner Mongolia Medical University. Small interfering RNAs (siRNAs) against OIP5 (OIP5-KD1 and OIP5-KD1) or negative control siRNAs (Control) were purchased from Transheep Co., Ltd. (Xuhui, Shanghai, China). The cell transfection was conducted by the use of Sinofection reagent (Sino Biological, Tongzhou, Beijing, China) in accordance with the manufacturer's protocols.

Reverse Transcription-Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the NPC cells by Ultrapure RNA kit (CWBIO, Changping, Beijing, China). First-strand cDNA synthesis was conducted using HiFiScript gDNA Removal cDNA Synthesis kit (CWBIO, Changping, Beijing, China). Subsequently, the qRT-PCR assays for OIP5 were carried out by LightCycler 480 II Real Time-Polymerase Chain Reaction System (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR RT-PCR kit (GenePharma, Shanghai, China). The expression of OIP5 was detected using GAPDH as an internal reference. All the primer sequences were listed in Table I.

Western Blot Analysis

The CNE1 and SUNE1 cells were washed twice with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA); then radioimmunoprecipitation assay (RIPA) cell lysis buffer (NeoBioscience, Shenzhen, Guangdong, China) containing protease inhibitor cocktail (AbMole Bioscience, Houston, TX, USA) was added. The protein concentration was examined by bicinchoninic acid (BCA) kit (Sigma-Aldrich, St. Louis, MO, USA). Afterward, the protein samples were mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and separated on 8%-12% SDS-PAGE.

Table I. Primer sets used in the present study.

Gene names	Sequences (5'-3')
OIP5 (Forward Primer)	TGAGAGGGCGATTGACCAAG
OIP5 (Reverse Primer)	AGCACTGCGTGACACTGTG
GAPDH (Forward Primer)	TGGCCTTCCGTGTTCTCTAC
GAPDH (Reverse Primer)	GAGTTGCTGTTGAAGTCGCA

Then, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Lab, Hercules, CA, USA). After the membranes were sequentially incubated with primary and secondary antibodies, the protein bands were evaluated by an ECL Western blotting substrate kit (Abcam, Cambridge, MA, USA) and a Tanon Gel Imaging System (Tanon, Minhang, Shanghai, China). The antibodies used in the present study were as followed: Vimentin and N-Cadherin (ProteinTech, Wuhan, Hubei, China), JAK2 and STAT3 (R&D Systems, Minneapolis, MN, USA), phosphorylated JAK (p-JAK2) and p-STAT3 (Cell Signaling Technology, Danvers, MA, USA).

Cell Viability and Colony Formation Assays

CNE1 or SUNE1 cells (approximately 2000 cells per well) were cultured in 96-well plates and kept at 37°C with 5% CO₂ in an incubator overnight. At the indicated timepoint, we added 10 µl of Cell Counting Kit-8 (CKK-8; MedChem Express, Monmouth Junction, NJ, USA) solution into each well. After incubating at 37°C with 5% CO₂ for an additional 2 h, the absorbance at 450 nm was examined by a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The colony formation assays were conducted by plating the CNE1 or SUNE1 cells (approximately 500 cells per well) in six-well plates, culturing for about two weeks and finally staining with 0.1% crystal violet solution.

Cell Apoptosis Assay

Cell apoptosis was detected by a FACSCalibur HG flow cytometer (BD, Franklin Lakes, NJ, USA) using Annexin V-EGFP/PI Apoptosis Detection kit (R&S Biotechnology, Shanghai, China). Briefly, after transfection of siRNAs (Control, OIP5-KD1 and OIP5-KD1), CNE1 or SUNE1 cells were collected and resuspended in the binding buffer. Then, Annexin V as well as propidium iodide (PI) were added into the cell suspensions and the cells were incubated for 15 min at room temperature. Finally, the flow cytometer was utilized to analyze the cell apoptosis.

Wound Healing Assays

After the CNE1 or SUNE1 cells were transfected with siRNAs (Control, OIP5-KD1 and OIP5-KD1), 70 µl of the cells (at a density of 5×10⁵ cells/ml) were into an Ibidi 3.5cm µ-Dish with culture insert (Thunder Scientific, Minhang, Shanghai, China). After 24 h, a sterile tweezer was applied

to remove the culture inserts and 500 µl of medium with 10% of fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) were added into the dish. The wounded areas were imaged by a microscope (YDF-90; YuanRen, Pudong, Shanghai, China) at 0 h and 24 h.

Transwell Assays

Briefly, CNE1 or SUNE1 cells transfected with siRNAs (Control, OIP5-KD1 and OIP5-KD1) were resuspended in the serum-free medium and then added into the upper chambers of a BD Biosciences transwell filter (8.0 µm pore size; Franklin Lakes, NJ, USA). The transwell filter was pre-coated with 50 µl of Matrigel, which was purchased from BD Biosciences Co., Ltd. (Franklin Lakes, NJ, USA). Then, 500 µl of medium with 20% fetal bovine serum (FBS) were added into the lower chamber. After incubation of 24 h, the invaded cells at the bottom of the chambers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. Finally, the images were photographed under a microscope (YDF-90; YuanRen, Pudong, Shanghai, China) after being washed with Phosphate-Buffered Saline (PBS) three times.

Statistical Analysis

All data were carried out using the SPSS 17.0 software package (version 17.0, SPSS Inc, Chicago, IL, USA). Data are presented as the mean ± SD. Statistical differences between groups were evaluated using the Student's paired two-tailed *t*-test. One-way ANOVA and Tukey post-hoc test was performed to analyze the difference among three or above groups. Statistical significance was presumed when *p* < 0.05.

Results

Integrative Bioinformatics Analysis Revealed that OIP5 Was Highly Expressed in NPC Tissues and Cell Lines

To characterize the aberrantly expressed genes in NPC, we first carried out comprehensive bioinformatics analysis by the use of two independent microarray datasets (GSE12452 and GSE53819) from Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/gds>). The bioinformatics analysis was performed using R package software. Heat maps were first generated based on the expression levels of differentially expressed genes (DEGs) in GSE12452 and GSE53819 datasets (Figure 1A).

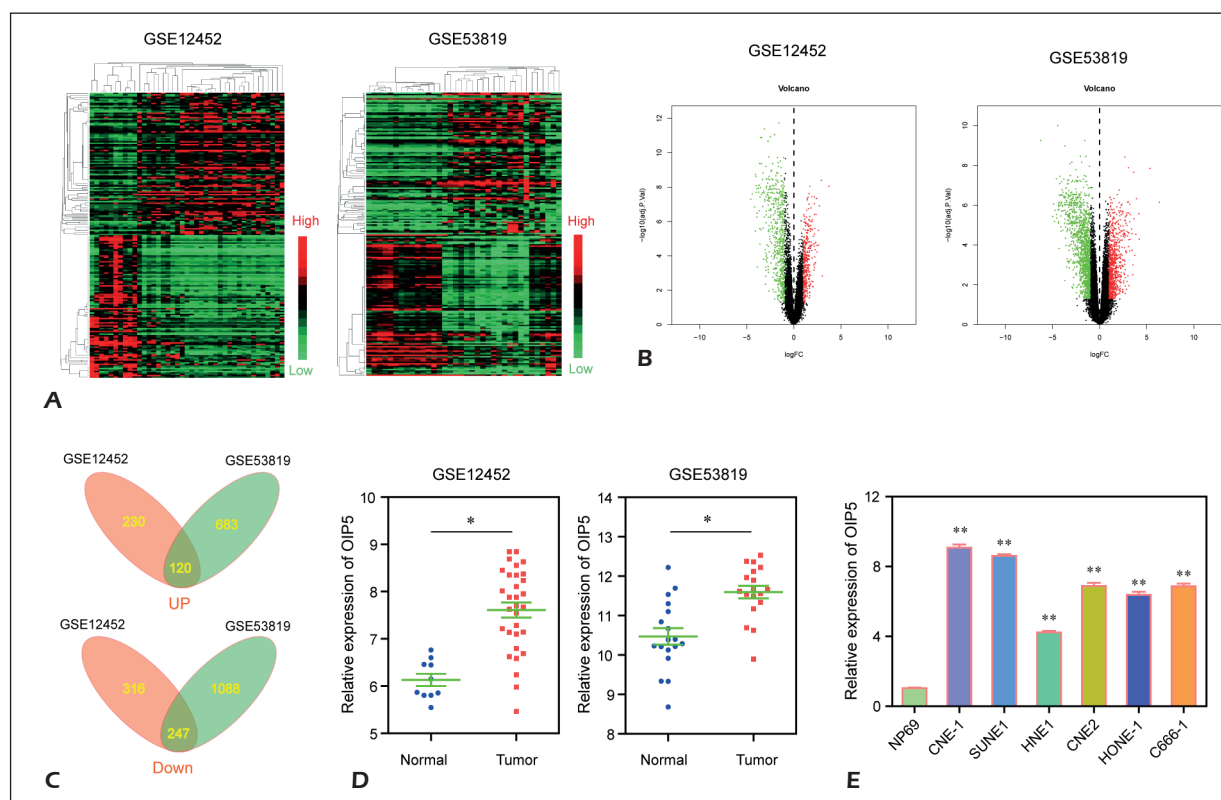


Figure 1. Bioinformatics analysis of differentially expressed genes in NPC tissues. **A**, Hierarchical clustering analysis of the differentially expressed mRNAs in GSE12452 and GSE53819. **B**, Aberrant expression mRNAs in GSE12452 and GSE53819 were reflected by the volcano plot. **C**, Venn diagram representation of the overlap of mRNAs that are differently expressed in two GEO datasets. **D**, The expression levels of OIP5 mRNA in NPC tissues and normal tissues from GSE12452 and GSE53819. **E**, The expression levels of OIP5 mRNA in NPC cell lines (SUNE-1, CNE-2, HONE-1, CNE-1, HNE-1 and C666-1) and nasopharyngeal epithelial cell line NP69 by RT-PCR. * $p < 0.05$, ** $p < 0.01$.

In addition, the distribution of DEGs between normal tissues and cancer tissues in GSE12452 and GSE53819 datasets were also exhibited by volcano plots (Figure 1B). Furthermore, Venn analysis suggested that 120 DEGs were consistently up-regulated and 247 DEGs were consistently down-regulated in both GSE12452 and GSE53819 datasets (Figure 1C). Besides, among the 120 consistently up-regulated DEGs, we selected OIP5, which was up-regulated in nasopharyngeal cancer tissues of GSE12452 and GSE53819 datasets for further studies because it might play important roles in NPC (Figure 1D). Moreover, qRT-PCR assays were also utilized to examine the expression levels of OIP5 in NPC cell lines. The data revealed that the expression levels of OIP5 were remarkably increased in NPC cell lines (CNE-1, SUNE1, HNE1, CNE2, HONE-1 and C666-1) compared with the control cells, NP69 (Figure 1E). Collectively, these results suggested that OIP5 was highly expressed in NPC tissues and cell lines.

Depression of OIP5 Suppressed NPC Cells Proliferation and Induced Cells Apoptosis

As the expression levels of OIP5 was elevated in NPC tissues and cell lines, we wondered whether OIP5 played an essential role in regulating the development of NPC. To achieve that, we performed loss-of-function studies to test the influence of OIP5 on cell proliferation and apoptosis in the following experiments. The qRT-PCR assays suggested that the expression levels of OIP5 were significantly decreased in CNE1 and SUNE1 cells transfected with OIP5-KD1 and OIP5-KD1 siRNAs (Figure 2A). Thereafter, the viability capabilities of CNE1 and SUNE1 cells were evaluated using CCK-8 assays. We observed that transfection of OIP5-KD1 and OIP5-KD1 siRNAs could dramatically suppress the growth of CNE1 and SUNE1 cells (Figure 2B). Besides, the colony formation abilities of CNE1 and SUNE1 cells were also remarkably reduced after knockdown of OIP5

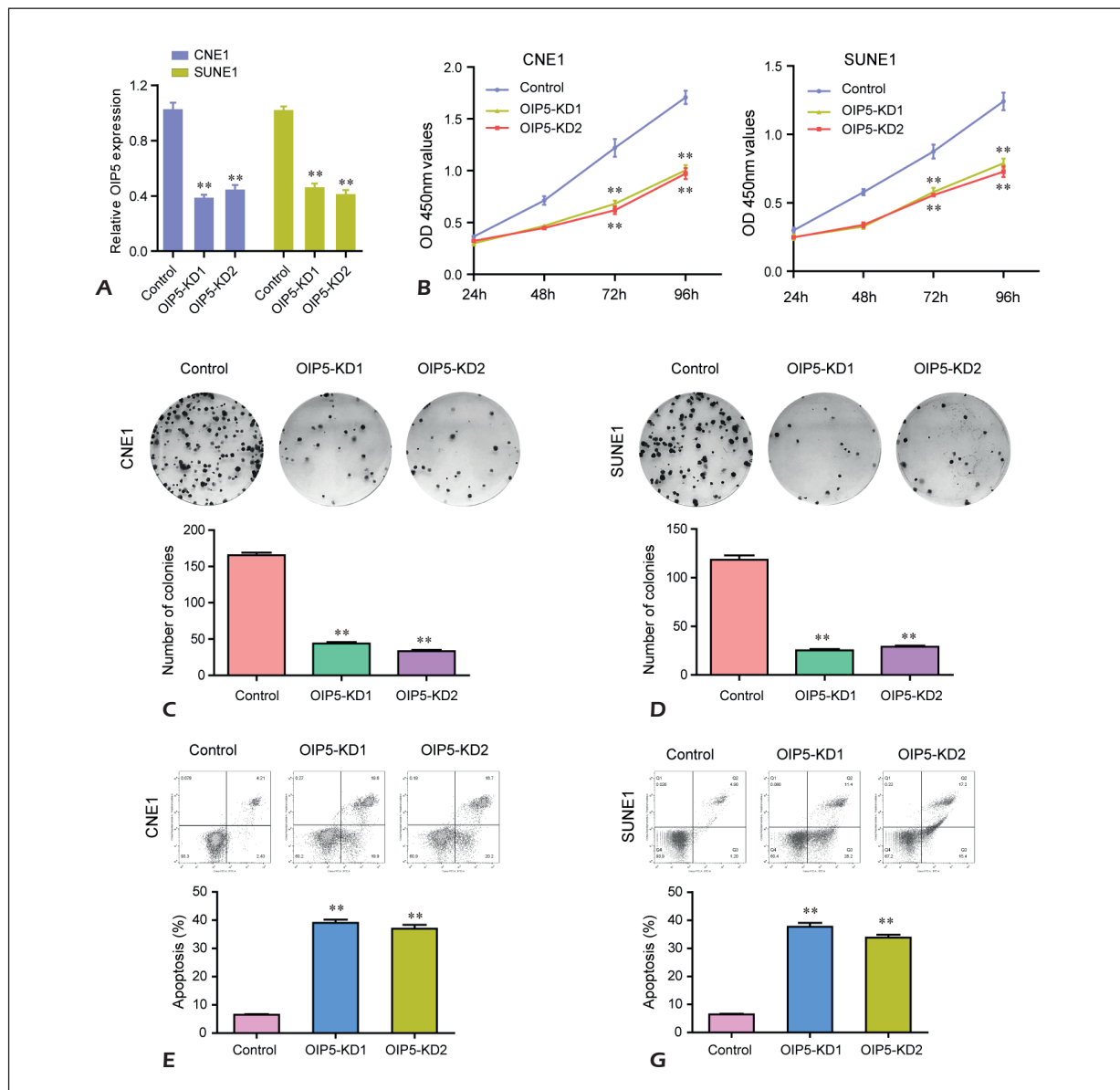


Figure 2. The effects of OIP5 on the proliferation and apoptosis of CNE1 and SUNE1 cells. **A**, Relative mRNA levels of OIP5 in CNE1 and SUNE1 cells transfected with OIP5 siRNAs (OIP5-KD1 and OIP5-KD2) or negative control siRNAs (Control). **B**, Transfection of OIP5 siRNAs suppressed the proliferation of CNE1 and SUNE1 cells which was examined by CCK-8 assays. **C-D**, Knockdown of OIP5 reduced colony formation capabilities of CNE1 and SUNE1 cells. **E-F**, Flow cytometry analysis evaluated the cells apoptosis analysis of CNE1 and SUNE1 cells transfected with OIP5 siRNAs or negative control siRNAs. * $p < 0.05$, ** $p < 0.01$.

(Figure 2C and D). In addition, we next conducted flow cytometry analysis to examine the apoptotic rates of CNE1 and SUNE1 cells. According to the data, transfection of OIP5-KD1 and OIP5-KD2 siRNAs led to a significant decline of apoptotic rates of CNE1 and SUNE1 cells (Figure 2E and F). These results indicated that silence of OIP5 could inhibit the development of NPC *in vitro*.

Silence of OIP5 Impaired the Metastatic Potentials of Nasopharyngeal Cancer Cells

Next, we focused on the influence of OIP5 on the metastatic potentials of nasopharyngeal cancer cells. Hence, wound healing and transwell assays were carried out using CNE1 and SUNE1 cells. As the results of wound healing assays showed, the wounded areas were remarkably wider in the

CNE1 and SUNE1 cells transfected with OIP5-KD1 and OIP5-KD1 siRNAs than that of the control cells (Figure 3A and B). In addition, transwell assays also revealed that depletion of OIP5 resulted in a significant decline of invasive cell number of CNE1 and SUNE1 cells (Figure 3C and D). As the suppression of OIP5 could markedly inhibit the migratory and invasive abilities of nasopharyngeal cancer cells, we next asked whether OIP5 could modulate the expression of epithelial-mesenchymal transition (EMT) related molecules, such as N-cadherin and Vimentin in CNE1 and SUNE1 cells. Thus, we performed Western blot assays to examine the protein levels of these molecules. The data demonstrated that the knockdown of OIP5 depressed the protein levels of N-cadherin and Vimentin in CNE1 as well as SUNE1 cells (Figure 3E). In summary, these results indicated that the knockdown of OIP5 could suppress the metastatic potentials of NPC.

Knockdown of OIP5 Suppressed the Activation of JAK2-STAT3

Signaling in Nasopharyngeal Cancer Cells

To further ascertain the underlying molecular mechanisms involved in the effects of OIP5 on the development as well as the progression of nasopharyngeal cancer cells, we next aimed to examine the protein levels of molecules involved in JAK2-STAT3 signaling which modulated the development and progression of various cancers. As the results of Western blot presented in Figure 4A, there was a remarkable decrease in protein levels of phosphorylated JAK2 (p-JAK2) and the phosphorylated STAT3 (p-STAT3) in CNE1 cells transfected with OIP5-KD1 and OIP5-KD1 siRNAs, though the protein levels of JAK2 as well as STAT3 were not changed. Analogously, notable repression of p-JAK2 as well as p-STAT3 was observed in SUNE1 cells when OIP5 was silenced by siRNAs (Figure 4B). These results confirmed that the suppressive effects of OIP5 knockdown on the proliferation, migration and invasion of nasopharyngeal cancer cells might be *via* abolishing the activity of the JAK2-STAT3 signaling pathway.

Discussion

NPC remains a serious health problem with a high incidence in South China¹⁹. The malignant transformation of the nasopharyngeal epithelial cell into metastatic NPC cells is the result of a process that requires a complex interaction^{20,21}. In

the last decade, although significance advancements have been achieved in understanding the potential molecular underlying NPC progression, the use of genetic alterations for diagnostic, prognostic, or therapeutic purposes remains limited^{22,23}. The identification of novel dysregulated genes would be of considerable importance for the screening and treatment of NPC. In this work, we first reported that OIP5 was one of the most up-regulated genes in NPC by analyzing GEO datasets (GSE12452 and GSE53819). Interestingly, a significant overexpression of OIP5 was observed in both two datasets *via* the results of the Venn diagram. Moreover, the results from RT-PCR by detecting the expression of OIP5 in several cells lines confirmed that OIP5 was highly expressed in NPC cell lines. Thus, our results revealed that the up-regulation of OIP5 may contribute to the progression of NPC. OIP5, a cancer-testis specific gene that was reported to be associated with cell cycle progression, has been found to be up-regulated in several tumors and act as a tumor promoter in tumorigenesis. For instance, OIP5 not only was highly expressed in bladder cancer but also acted as a prognostic and diagnostic biomarker in bladder cancer patients¹³. In addition, oncogenic role of OIP5 was also confirmed *in vitro*. Li et al²⁴ referred that OIP5 was overexpressed in hepatocellular carcinoma, especially in advanced hepatocellular carcinoma, and its knockdown could suppress the proliferation and migration in hepatocellular carcinoma cells by modulating the AKT/mTORC1 and β -catenin signaling pathways. These results indicated that oncogene OIP5 may play an important role in cancer proliferation and metastasis. However, the biological function of OIP5 in NPC has not been investigated. In this work, in order to explore the role of OIP5 in NPC, NPC cells with OIP5 knockdown were established. The functional investigation indicated that the down-regulation of OIP5 significantly suppressed NPC cells proliferation, migration and invasion, and promoted apoptosis, which revealed that OIP5 may be a treatment targeting for NPC. Thus, our data, together with previous studies, indicated that OIP5 may act as a common positive regulator in cancer progression. Janus kinase (JAK) signal transducer and activator of transcription (STAT) signal pathway are responsible for many cellular functions, such as proliferation, cell differentiation and cell apoptosis^{25,26}. Activated STAT3 was the crucial contributor to the proliferation of tumor cells. Recently, more and more studies focused on the

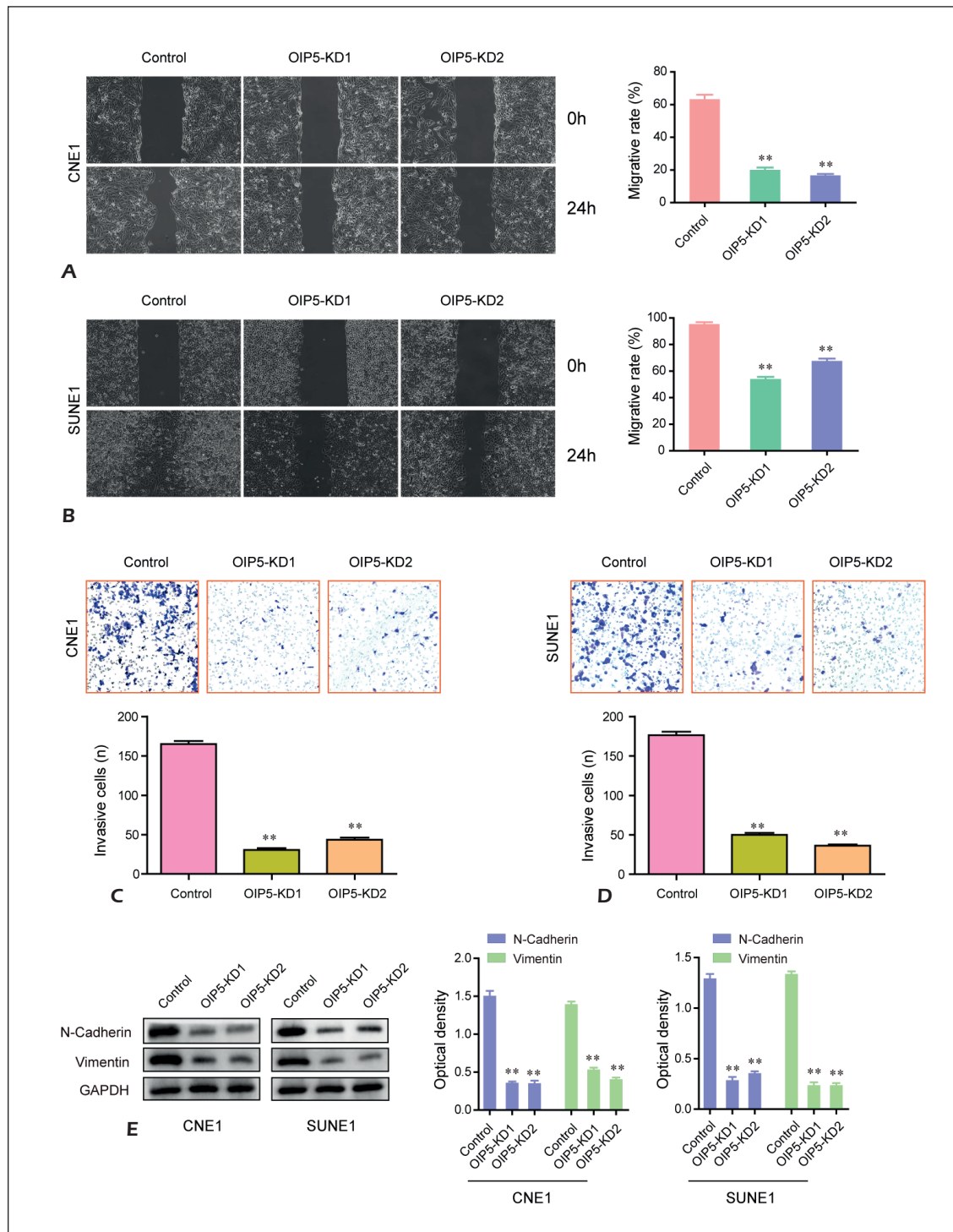


Figure 3. Silence of OIP5 affected the migration and invasion of CNE1 and SUNE1 cells. **A-B**, Migration capacities was measured by wound healing assays and transfection of OIP5 siRNAs (OIP5-KD1 and OIP5-KD1) significantly reduced the migratory capacities of CNE1 and SUNE1 cells. **C-D**, The invasive abilities of CNE1 and SUNE1 cells transfected with OIP5 siRNAs were notably decreased compared with the control cells. **E**, The protein levels of N-cadherin and Vimentin were significantly reduced in CNE1 and SUNE1 cells transfected with OIP5 siRNAs. * $p < 0.05$, ** $p < 0.01$.

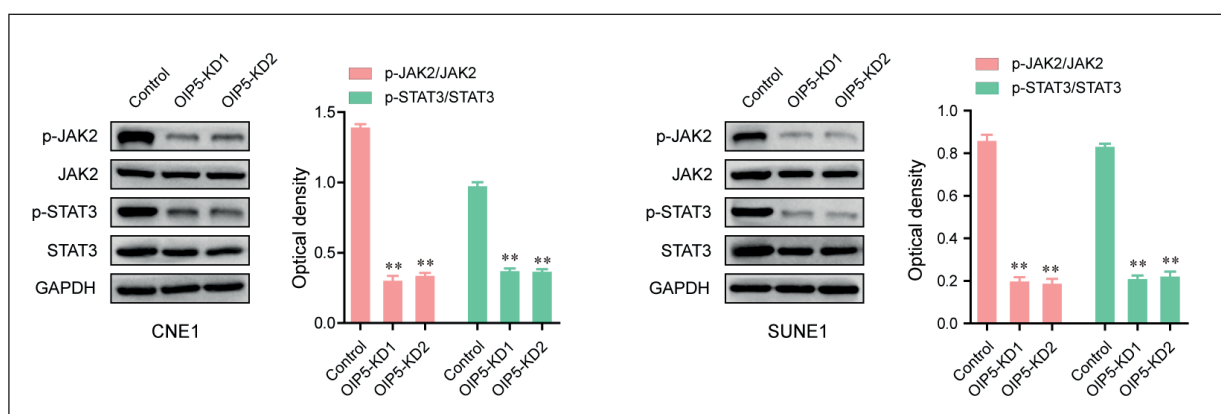


Figure 4. OIP5 modulated the activity of JAK2-STAT3 signaling in CNE1 and SUNE1 cells. **A**, Western blot assays were utilized to examine the protein levels of p-JAK2, JAK2, p-STAT3 and STAT3 in CNE1 cells and the relative optical density of these protein bands were analyzed by Image J software (version 1.46; NIH, Bethesda, MD, USA). **B**, The protein levels and optical density analysis of p-JAK2, JAK2, p-STAT3 and STAT3 in SUNE1 cells. * $p < 0.05$, ** $p < 0.01$.

specific roles of JAK2/STAT3 signal, finding that this pathway was involved in the regulation of various tumors, including NPC^{27,28}. On the other hand, the upstream mechanism of JAK2/STAT3 has also been studied: several tumor-related genes displayed their oncogenic or anti-oncogenic role by modulating JAK2/STAT3²⁹⁻³¹. In this work, we wondered whether OIP5 exhibited its role of tumor promoter by regulating JAK2/STAT3. To demonstrate it, we performed Western blot to explore the potential role of OIP5 knockdown in the activation of JAK2/STAT3, finding that OIP5 knockdown reduced the expression of phosphorylated STAT3 and phosphorylated JAK2 in both CNE1 and SUNE1 cell lines. Thus, our findings revealed that OIP5 displayed its anti-metastatic role probably by modulating the JAK2/STAT3 pathway in NPC cells.

Conclusions

We showed that OIP5 promoted NPC proliferation, metastasis and EMT by modulating the JAK2/STAT3 pathway. These findings imply that OIP5 might be a suitable candidate for anticancer therapy. In view of its potential clinical value, a thorough study into it is worth the effort.

Conflict of Interests

The authors declare that there are no conflicts of interest.

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