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LASP2 functions as a potential prognostic factor and therapeutic target in nasopharyngeal carcinoma

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the potential effects of LIM and Src homology 3 (SH3) protein 2 (LASP2) on nasopharyngeal carcinoma (NPC) and the relevant mechanism.

PATIENTS AND METHODS: The expression of LASP2 in NPC patients and non-cancer patients in the control group was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The patients were divided into LASP2 high-expression group (n=30) and low-expression group (n=30), according to the median expression level of LASP2. Then, the expression of LASP2 was detected in the chosen cell lines by qRT-PCR.

RESULTS: In qRT-PCR experiment, LASP2 was found up-expressed in NPC clinical samples and cell lines. Besides, LASP2 expression was associated with the clinical stage and distant metastasis of NPC. Next, the expression of LASP2 was downregulated by transfection of si-LASP using Lipofectamine™ 3000 in 6-10B cells *in vitro*. The transfection effects of si-LASP2 were confirmed by qRT-PCR and Western-blot (WB) experiments. In supplementary experiments, decreased expression of LASP2 in cells could inhibit the cell biological functions, including invasion, migration, and epithelial-mesenchymal transition (EMT).

CONCLUSIONS: This research discovers the promotion effect of LASP2 on NPC, suggesting that LASP2 could be used as a potential therapeutic target for NPC.

Key Words:

LIM and Src homology 3 protein 2, Nasopharyngeal carcinoma, Epithelial-mesenchymal transition.

Introduction

Nasopharyngeal carcinoma (NPC), a common head and neck malignancy prevalent in Southeast Asia[1,2], has attracted more attention than other head and neck malignancies due to its strong invasiveness and metastasis[3]. NPC patients are sensitive to radiotherapy, and their 5-year survival rate after radiotherapy is 50-60%, while local recurrence and distant metastasis are the leading causes of treatment failure[4]. Currently, the prognosis of NPC patients is evaluated mainly based on tumor-node-metastasis (TNM) stage, but studies have manifested that the clinical outcomes of patients with the same clinical stage are often different. It is inadequate to predict the prognosis of NPC based on the TNM stage[5]. Given this, studying the pathogenesis and new molecular markers of NPC might be conductive to early diagnosis and prognosis evaluation of NPC.

LIM and Src homology 3 (SH3) protein 2 (LASP2), originally predicted *via* bioinformatics, is located on human chromosome 10p12.31[6], which, like LASP1, belongs to the nebulin family. As to the structure of the two genes, a LIM domain is located at the amino terminus, and a SH3 domain at the carboxy terminus. Besides, recent research on LASP2 has focused on the functions related to the adhesion and movement of cells. LASP1, an important structural protein of cytoskeleton, is closely correlated with cell movement, which has been reported in many studies to have a close association with the development and progression of various malignant tumors, like metastatic breast cancer[7] and esophageal squamous cell carcinoma[8]. The distinction between LASP2 and LASP1 is the different number of nebulin-like repeat sequences in the structure. As a result, the specific role of LASP2 in tumors had become the research direction concerned in this study.

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Tumor metastasis refers to a complex and multi-step biological process, in which tumor cells infiltrate to distant organs and tissues from the primary site through local infiltration and vascular invasion so as to trigger colonization and tumorigenesis[9]. In addition, tumor metastasis results in over 90% of the death of cancer patients. Epithelial-mesenchymal transition (EMT), one of the most important mechanisms in local invasion and distant metastasis of epithelial-derived tumor cells, is a complex procedural biological process in which polar epithelial cells are transformed into mesenchymal cells under the co-participation and influence of many molecules, which plays an important role in normal life processes and is able to promote stem cell formation and wound healing[10]. Besides, EMT is mainly featured by the loss of such cell epithelial properties as cell-cell interactions and cell-matrix interactions of the basement membrane in this biological process, resulting in the loss of cell polarity. The expression of epithelial markers in cells declines, and that of mesenchymal markers rises, changing cell morphology, adhesion ability, and exercise capacity, leading to resisted apoptosis and enhanced secretion of degrading enzymes to invade extracellular matrix (ECM), and thereby gaining the property of invasiveness[11]. In recent years, Brabletz et al[12], Xiang et al[13] and Matysiak et al[14] have reported that EMT participates in tumor metastasis and is one of the important mechanisms promoting tumor progression.

In this research, the expression of LASP2 in NPC tissues and cell lines was investigated for the first time, and the role of LASP2 in cell function was further studied, in order to provide new and reliable potential therapeutic targets for the diagnosis and treatment of NPC.

Patients and Methods

Clinical Samples

A total of 60 patients pathologically diagnosed with NPC in our hospital from May 2016 to December 2018 were enrolled. As to tumor staging, the staging methods jointly developed by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) were adopted. Inclusion criteria: 1) patients admitted to the Department of Oncology in our hospital for surgical treatment and pathologically diagnosed with NPC; 2) those with sufficient tissue paraffin specimens stored in the Pathology Department of the hospital for further experiments, complete clinical data, and available contact information; 3) those receiving no neoadjuvant chemotherapy and neoadjuvant targeted therapy before surgery, but undergoing preoperative immunotherapy. Other 40 cases of nasopharyngeal mucosa confirmed as chronic inflammation by pathology in the same period were selected as controls. All patients were informed of this study that was approved by the Clinical Ethics Committee of our hospital and signed the informed consent.

Cell Culture and Transfection

Human immortalized nasopharyngeal epithelial cell line (NP69) and human NPC cell line (6-10B) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Human NPC cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and human immortalized nasopharyngeal epithelial cell lines were cultured in KSFM and EpiLife (1:1; Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 at 37°C. The cells were transfected according to the instructions of the Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) when the cell density reached 50-60%.

Groups were divided as: NC group (6-10B cells transfected by si-NC) and LASP2 group (6-10B cells transfected by si-LASP2).

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

The total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the expression level of LASP2 and controlled by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences of LASP2 were as follows: sense primer 5'-CATTCCCAAGGCTATGGCTA-3' and anti-sense primer 5'-ATCGTACATGGCTCGGTAGG-3'. The primer sequences for GAPDH were as follows: sense primer 5'-CCACCCATGGCAAATTCCATGGCA-3' and anti-sense primer 5'-TCTAGACGGCAGGTCAGGTCCAC-3'.

Western Blot Analysis

Cell samples were collected at 48 h after transfection and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein concentration in cells was de-

tected using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). A total of 50 μg of protein was loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% separating gel, wet transferred onto a membrane at 300 mA for 100 min and sealed with 5% skim milk powder solution at 37°C for 1 h. The membrane was washed using Tris-Buffered Saline with Tween-20 (TBST) for 3 times (10 min/time), incubated with LASP2, E-cadherin, N-cadherin, Vimentin primary antibody or GAPDH antibody (1:1000) overnight at 4°C and incubated again with the corresponding secondary antibody at room temperature for 1 h. After the membrane was washed using TBST for 3 times, the samples were exposed in a darkroom.

Cell Invasion and Migration

At 48 h after cell transfection, the cells were resuspended in the serum-free DMEM and the concentration was adjusted to 2×10^5/L. A total of 600-800 μL of culture medium containing 10% FBS was added into the lower transwell chamber and 100-150 μL of cell suspension into the upper chamber. Subsequently, the cells were continuously cultured for 24 h, then, fixed with paraformaldehyde for 30 min, stained with 0.1% crystal violet for 20 min and observed under an inverted microscope. Next, five random fields of vision were selected to count the cells and the statistical results were taken. In the invasion experiment, the transwell chamber did not need to be coated with Matrigel and the rest procedures were the same as those in the migration assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The differences between the two groups were analyzed by using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Chi-square test was performed for the association between 6-10B and clinicopathological parameters of NPC patients. $p<0.05$ was considered statistically significant.

Results

LASP2 was Upregulated in NPC

The expression of LASP2 in NPC patients and non-cancer patients in the control group was detected by qRT-PCR, the expression of LASP2 in NPC patients was made as reference (1.685 ± 0.414), LASP2 showed a high expression state in NPC patients (5.372 ± 1.105) (Figure 1). The result suggests that LASP2 may be an effective tumor-promoter in NPC.

The Relationship of LASP2 With Clinicopathological Features

The patients were divided into LASP2 high-expression group (n=30) and low-expression group (n=30) according to the median expression level of LASP2. The association between LASP2 expression and clinicopathological features of patients was analyzed, as shown in Table I. Excluding gender, age, T classification, and N classification, LASP2 was found to be positively associated with clinical stage and distant metastasis of NPC.

Si-LASP2 Decreased the Expression Level of LASP2

The expression of LASP2 was detected in the chosen cell lines by qRT-PCR. LASP2 also showed a significant upward trend in NPC cells, which was the same as we expected (Figure 2A).

From the point of view of RNA and protein expressions, PCR and WB assays were performed after transfection and it could be found that the expression of LASP2 was decreased in si-LASP2 treated 6-10B cells by comparing with that in the si-NC treated cells (Figure 2B).

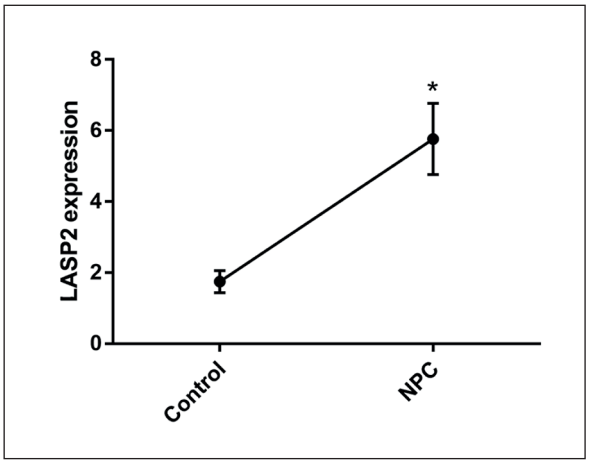

Figure 1. The expressions of LASP2 in NPC patients and normal control patients (*$p<0.05$).

Table I. LASP2 expression and clinical features of patients with NPC.

Variable		LASP2 expression		*p*-value
		High	Low	
No.	60	30	30	
Gender				0.790
Female	14	6	8	
Male	46	20	26	
Age (years)				0.874
< 45	24	11	13	
≥ 45	36	16	20	
T classification				0.193
T1-T2	26	10	16	
T3-T4	34	20	14	
N classification				0.589
N0-N1	21	9	12	
N2-N3	39	21	18	
Clinical stage				0.019*
I-II	26	8	18	
III-IV	34	22	12	
Distant metastasis				0.026*
Yes	19	14	5	
No	41	16	25	

Si-LASP2 Inhibited the Invasion and Migration of NPC Cells

The results from the transwell assay suggested that si-LASP2 could effectively inhibit the migration and invasion of 6-10B cells, and the number of transmembrane cells was significantly reduced in the LASP2 group (Figure 3A), demonstrating that promoting effects of LASP2 on invasion and migration of NPC cells were confirmed. Besides, EMT-related markers were detected, and the similar results were obtained in WB experiment that si-LASP2 limited the expression of mesenchymal

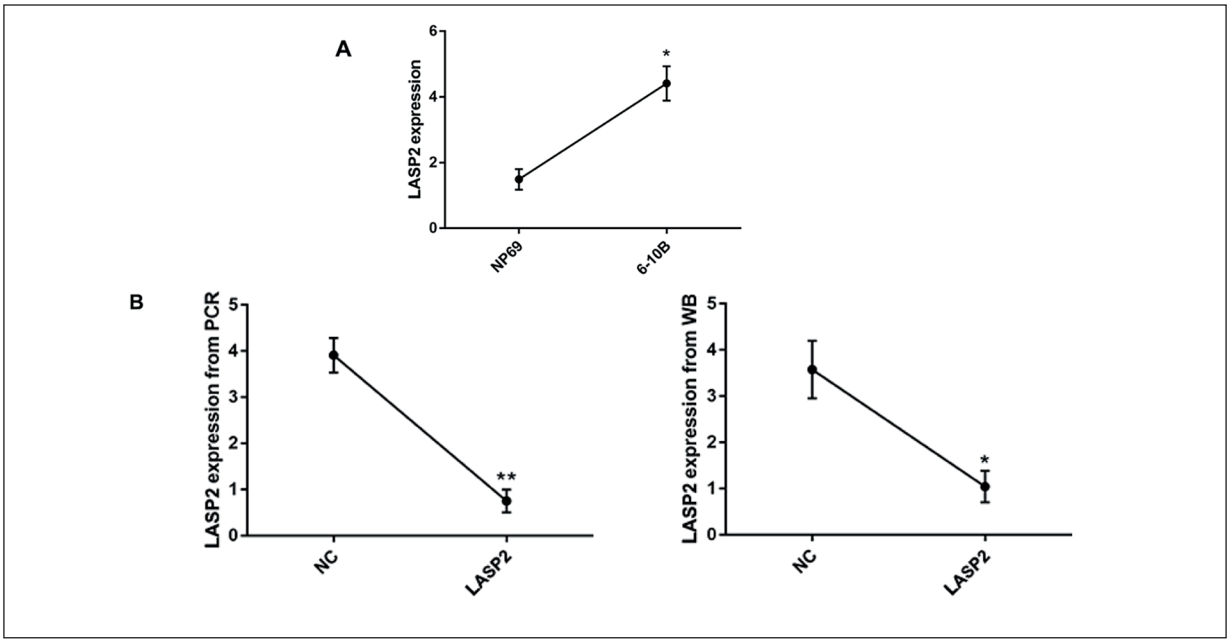

Figure 2. A, The expressions of LASP2 in NPC cells and reference normal cells (*p<0.05). **B,** The expression of LASP2 in 6-10B cells tested by qRT-PCR and Western blot analysis (*p<0.05, ** p<0.01).

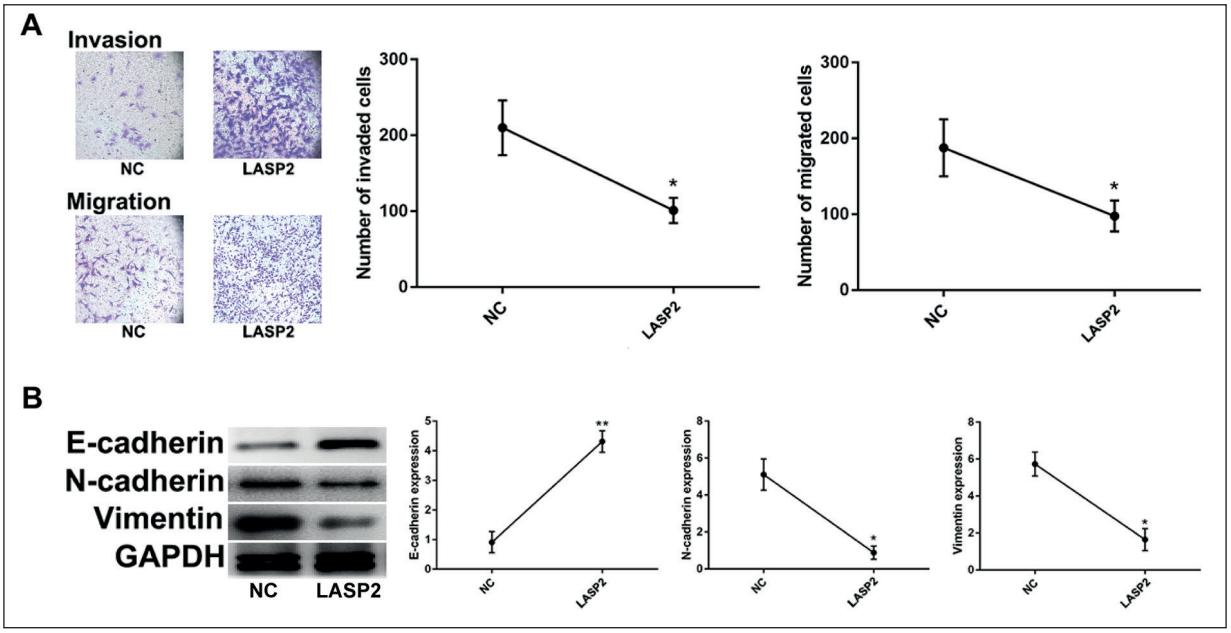

Figure 3. A, si-LASP2 inhibits the invasion and migration of NPC cells (magnification: 400×) (*p<0.05). **B,** si-LASP2 affects the expression level of EMT-related protein detected by Western blot experiment (*p<0.05, ** p<0.01).

phenotype protein (N-cadherin and Vimentin) while recovering the expression of epithelial phenotype protein (E-cadherin) (Figure 3B). These experimental data partially explained the high correlation between LASP2 and distant metastasis of NPC in clinical samples.

Discussion

The LIM and SH3 domains in the structure of the LASP2 protein interact with various cytoskeletal proteins and signaling pathway proteins, thus affecting cytoskeletal formation, cell adhesion, cell movement, and signal transduction[15-17]. Existing studies of LASP2 have reported that LASP2, as an actin-binding protein, is a part of focal adhesion complexes and interacts with zyxin in the actin bundles of filopodia and lamellipodia in nerve cells, which promote the adhesion and spreading of fibroblasts and can be recruited to the focal adhesion front of the stimulated cells. Meanwhile, it is also discovered that in HeLa cells and NIH3T3 cells, LASP2 co-localizes with a-catenin, and the expression of LASP1 is absent in these regions. Moreover, the overexpression of LASP2 in PC6 cells cultured under the stimulation from growth factors inhibit neurite hyperplasia[15,18,19]. Additionally, LASP2 interacts with its binding proteins (vinculin and paxillin) and is capable of enhancing the interaction between vinculin and paxillin. Nevertheless, it is interesting that the presence of LASP1 weakens these interaction[20]. These research findings indicate that LASP2 is an important participant in cytoskeletal formation, cell adhesion, cell movement, and intercellular communication, whose role is completely different from that of LASP1 in cytoskeletal proteins.

Tumor cells undergo EMT under the combined action of various factors, losing the epithelial property linked to the basement membrane and thus obtaining mesenchymal property that is relatively radical, and then, they enter the surrounding matrix through multiple biological processes to enter the lymphatic and blood systems and colonize them, eventually resulting in tumor metastasis[21,22]. Currently, EMT is deemed as a hierarchical and dynamic process, which plays a major role in the early process in which tumor cells escape from the primary site of the tumor, enter the vascular system through the extracellular matrix, and reach distant metastatic organs and tissues following the blood circulation and lymphatic

circulation, while after colonization of tumor cells, MET occurs[23].

The experimental results of this study revealed for the first time the role of LASP2 in NPC. Similar to the cancer-promoting effect of LASP2 reported in other tumors[24,25], LASP2 exhibited high expression in NPC patients and was associated with the clinical stage and distant metastasis of NPC. When the expression level of LASP2 in NPC cells was artificially decreased *in vitro*, the migration and invasion of NPC cells were also significantly reduced, and the effect of si-LASP was also reflected in the inhibition of EMT.

Conclusions

In summary, the results of this study provide a new potential molecular therapeutic target for treatment of NPC. However, the role of LASP2 in NPC needs further exploration.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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