

AIG3 Induces AURKA to Promote Laryngeal Squamous Cell Carcinoma Metastasis

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Abstract

Objectives To investigate the relationship between ALG3 and AURKA, the expression and potential prognostic value of ALG3 in LSCC, and to then explore the impact of ALG3 in tumorigenic effects.

Methods Co-immunoprecipitation assay was detected the relationship between ALG3 and AURKA, Rt-PCR and Western blot was detected the expression of related mRNA and proteins. CCK8 assay, plate colony formation assay Cells, wound healing, migration and invasion assays were used to examine the ability of proliferation, movement, migration and invasion of LSCC cells.

Results ALG3 immediately induced AURKA to promote LSCC metastasis. Moreover, ALG3 highly expressed in LSCC tissues and cells and the expression of ALG3 was positively related to tumor size, lymphatic metastasis and poor clinical prognosis. Furthermore, knockdown ALG3 in LSCC cells remarkably restrained cellular proliferation, migration and invasion *in vitro* and *in vivo*.

Conclusion ALG3 induced AURKA to promote LSCC metastasis and ALG3 maybe potential prognostic value for LSCC.

Brief Abstract

ALG3 induces AURKA to promote laryngeal squamous cell carcinoma metastasis

Introduction

Laryngeal squamous cell carcinoma LSCC is one of the most common subtype of Laryngeal carcinoma¹. It is also the type of Laryngeal carcinoma that has the highest rate of mortality and morbidity². Radiotherapy, chemotherapy and surgery, alone or in combination were applied to LSCC treatment³. However, the 5-year overall survival (OS) rate was 63%⁴. LSCC was difficult to be completely cured due to tumor metastasis. However, specific molecular mechanism of LSCC metastasis remained unclear.

Our previous research found Aurora kinase A (AURKA) revives dormant LSCC to promote metastasis^{3,5}. Therefore, illustrating specific molecular mechanism of AURKA revives dormant LSCC to promote metastasis was of great concern. The key molecules maybe capable to potential prognostic value and potential targets for clinical LSCC treatment.

Alpha-1, 3-mannotransferase (ALG3), located on the chromosomal region 3q27.1, was an oncogene implicated in multiple malignancies, for instance, non-small cell lung cancer⁶, breast cancer⁷, oral squamous cell carcinoma⁸, acute myeloid leukemia⁹, et al.

ALG3, related with early N-glycans synthesis, was located in the endoplasmic reticulum and Golgi apparatus¹⁰. ALG3 was conducive to high-mannose type N-glycans, which promoted cancer

progression¹¹. AURKA was reportedly regulated architecture of the Golgi apparatus¹². Therefore, we guess whether ALG3 got in touch with AURKA.

In our study, we first investigated the relationship between ALG3 and AURKA, and then ALG3 expression level was examined in LSCC tissues and cells. Afterwards, the correlations between ALG3 and clinical features and potential prognostic value were analyzed. Furthermore, the expression of ALG3 was regulated in LSCC cells. We found ALG3 got in touch with AURKA. Moreover, ALG3 highly expressed in LSCC tissues and cells and the expression of ALG3 was related to tumor size, lymphatic metastasis and poor clinical prognosis. Furthermore, knockdown ALG3 in LSCC cells remarkably restrained cellular proliferation, migration and invasion.

Results

1. ALG3 corelated with AURKA, ALG3 may directly induced AURKA to promote LSCC metastasis.

Our previous research found AURKA revives dormant LSCC to promote metastasis. Illustrating specific molecular mechanism of AURKA revives dormant LSCC to promote metastasis was of great concern. ALG3 was an oncogene implicated in multiple malignancies. Therefore, Co-IP assay was applied to explore the relationship between ALG3 and AURKA (Figure 1A and 1B). The result demonstrated that ALG3 corelated with AURKA. While, the regulatory mechanism between ALG3 and AURKA was ambiguous. Plasmid construction and transfection were applied in down-regulating the expression of ALG3 in TU686 cells (TU686/sh-ALG3), and a inhibitor (VX680¹³) was applied in down-regulating the expression of AURKA in TU686 cells (TU686/VX680). Western blot was used to test the effect of down-regulation. Results of figure 1C and 1D were shown that p-ALG3 and p-AURKA levels were downregulated obviously (*P<0.05, **P<0.01). Results of figure 1E and 1F were shown that p-AURKA level was reduced distinctly, while p-ALG3 level was not changed (**P<0.01). These results suggested that ALG3 corelated with AURKA, ALG3 was the upstream molecular of AURKA and ALG3 may directly induce AURKA to promote LSCC metastasis.

2. Overexpression of ALG3 in LSCC tissues and cells

In order to explore the role of ALG3 in promoting the metastasis of LSCC, the expression of ALG3 was investigated between LSCC tissues and matched adjacent non-tumor tissues. qRT-PCR was applied to assess the expression level of ALG3 in 30 LSCC patients. Results of qRT-PCR was shown that the mRNA level of ALG3 was observably

higher in tumor tissues than non-tumor tissues (**P<0.01, Figure 2A and 2B). Furthermore, ALG3 mRNA and protein levels were examined in LSCC cells line (Hep2, NH8, TU686, TU212) and cell line (Hela). The results shown that mRNA and protein levels of ALG3 were higher in Hep2, NH8, TU686 and TU212 cells, whereas lower in Hela cells. Those findings exhibited that ALG3 was upregulated in LSCC tissues and cells. Nevertheless, the high expression of ALG3 was related with clinicopathological features remained unknown.

3. ALG3 high expression level was related to clinicopathological characteristics and poor survival in LSCC patients

Chi-square test was applied to analyze relationship between ALG3 expression level and clinicopathological features of 60 LSCC patients was exhibited in Table 1. The results shown that ALG3 high expression level was observably related to tumor size ($P=0.037$), invasion range ($P=0.014$), lymph node involvement ($P=0.032$) and TNM stage ($P<0.001$). But there were no statistically significant relationships between ALG3 expression and other clinicopathological characteristics such as age ($P=0.223$) or gender ($P=0.874$). 60 LSCC patients were followed up by 5 years, which 27 patients had died. The mortality was 45% (27 of 60). The median survival time of 36 months for patients with strong ALG3 staining, while the median survival time of 49 months for patients with weak staining ($P=0.0398$). Above observations indicated that ALG3 high expression level was related to clinicopathological characteristics, poor survival and LSCC malignancy.

4. ALG3 promoted LSCC cellular ability of proliferation migration and invasion

ALG3 was highly expressed in LSCC tissues and cells, and ALG3 high expression level was related to clinicopathological characteristics and poor survival in LSCC patients. Hence, CCK8, plate colony formation and cell migration and invasion assays were performed to evaluate the ability of proliferation, migration and invasion of TU686 and NH8 cells. TU686/sh-ALG3 and NH8/sh-ALG3 cells reduced cell proliferation compared with the control groups respectively (TU686/vector and NH8/vector cells, Figure 4A and 4B $*P<0.05$, $**P<0.01$). Colony formation assays showed that the ability of proliferation was enhanced in TU686/vector (80 ± 7.23) and NH8/vector cells (62 ± 3.21) compared with TU686/sh-ALG3 (33 ± 4.54) and NH8/sh-ALG3 cells (24 ± 3.86 , Figure 4C, 4D, 4E and 4F, $**P<0.01$). Similarly, more TU686/vector (319 ± 10.26) and NH8/vector cells (240 ± 15.8) migrated through transwell chambers compared with TU686/sh-ALG3 (150 ± 8.51) and NH8/sh-ALG3 cells (172 ± 8.7 , Figure 4G, 4H, 4I and 4J, $**P<0.01$). Finally, invasion assays indicated that TU686/vector (51 ± 2.91) and NH8/vector cells (54 ± 4.4) moved through matrigel more frequently than TU686/sh-ALG3 (25 ± 3.28) and NH8/sh-ALG3 cells (20.7 ± 5.0 , 4G, 4H, 4I and 4J, $**P<0.01$). Above observations indicated that ALG3 overexpression could enhance cellular ability of proliferation, migration and invasion to promote LSCC metastasis.

5. Blocking ALG3 impaired LSCC metastasis in vivo.

In our study, we further estimated the contribution of ALG3 on LSCC metastasis in vivo. TU686/vector cells, NH8/vector cells, TU686/sh-ALG3 cells and NH8/sh-ALG3 cells were inoculated via tail vein into the mice. After six months, counting pulmonary metastatic nodules by H&E staining. As showed in Figure 5A-5B TU686/vector cells (4 ± 0.6) verified larger and more frequently lung metastases with respect to TU686/sh-ALG3 cells (6.3 ± 0.9 , $*P<0.05$). NH8/vector cells (0.7 ± 0.3) attested larger and more frequently lung metastases with respect to NH8/sh-ALG3 cells (1.0 ± 0.6 , $*P<0.05$). Results above suggested that blocking ALG3 impaired LSCC metastasis in vivo.

Discussion

Tumor metastasis accounts for the majority of cancer-related deaths worldwide and the revival of dormant tumor cells may be one of the mechanisms related to metastasis. However, the precise molecular and cellular regulators involved in this transition remain poorly understood¹⁴. Aurora kinase A (AURKA), the family of serine/threonine kinases, regulated the process of mitosis, which is necessary for cell division processes¹⁵. AURKA is frequently amplified and/or overexpressed in GI malignancies (including esophageal, gastric, and colorectal and head and neck cancers)¹⁶⁻¹⁹. Our previous research found Aurora kinase A (AURKA) revives dormant LSCC to promote LSCC metastasis^{3,5}. Therefore, illustrating specific molecular mechanism of AURKA revives dormant LSCC to promote LSCC metastasis was of great concern. The key molecules may be capable to potential prognostic value and potential targets for clinical LSCC treatment.

ALG3 was an oncogene implicated in multiple malignancies. ALG3 plays an important role in mitotic recombination²⁰. Furthermore, ALG3 was conducive to high-mannose type N-glycans, which promoted cancer progression¹¹. AURKA was reportedly regulated architecture of the Golgi apparatus¹². Therefore, we hypothesize whether AURKA interacts with ALG3 to promote the metastasis of LSCC.

In our study, we first investigated the relationship between ALG3 and AURKA. We guess if ALG3 is related to AURKA to promote LSCC metastasis. Results of Co-IP assay indicated that ALG3 correlated with AURKA. To further explore the regulatory mechanism between ALG3 and AURKA. Plasmid construction and transfection were applied in down-regulating the expression of ALG3 in TU686 cells, and the inhibitor (VX680) was applied in reducing the expression of AURKA in TU686 cells. Western blot was used to explore the effect of knock-down. Results suggested that ALG3 correlated with AURKA, ALG3 was the upstream molecular of AURKA and ALG3 may direct regulation AURKA to promote LSCC metastasis.

And then qRT-PCR and Western Blot were carried out to assess the ALG3 expression level in LSCC tissues and cells. ALG3 was upregulated in LSCC tissues and cells. To further illustrate the correlation between ALG3 and laryngeal cancer, the correlations between ALG3 and clinical features and potential prognostic value were analyzed.

Chi-square test was applied to analyze relationship between ALG3 expression level. The results indicated that ALG3 high expression level was observably related to tumor size, invasion range, lymph node involvement and TNM stage. 60 LSCC patients were followed up by 5 years, patients with high expression of ALG3 have a high mortality rate.

According to the above findings, ALG3 may function as an oncogene, causing malignant progression in LSCC. In order to further explore ALG3 plays a crucial role in the occurrence and development of laryngeal cancer. The expression of ALG3 was regulated in LSCC cells. Plasmid construction and transfection were applied in down-regulating the expression of ALG3 in TU686 and NH8 cells. Knockdown ALG3 in TU686 and NH8 cells remarkably restrain cellular ability of proliferation, migration and invasion in vitro and vivo.

In conclusion, our studies indicated that ALG3 correlated with AURKA, ALG3 may directly reduce AURKA to promote LSCC metastasis. Overexpression of ALG3 in LSCC tissues and cells. ALG3 high expression level was related to clinicopathological characteristics and poor survival in LSCC patients. And then ALG3 promoted LSCC cellular ability of proliferation, migration and invasion. Taken together, our findings indicate that ALG3 induces AURKA to promote laryngeal squamous cell carcinoma metastasis. And ALG3 represent potential targets for clinical LSCC treatment.

Materials And Methods

Patient samples and Ethical statement

LSCC tumor and non-tumor samples were collected from 30 patients between 2014 and 2021 at the Ruijin Hospital and Ninth hospital and Shanghai Pudong Gongli Hospital, Shanghai, China. This study was approved by the Human Research Ethics Committee of Pudong Gongli Hospital.

Cell lines

LSCC cells line (Hep2, NH8, TU686, TU212) and cell line (Hela) were preserved by the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Hep2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco company, USA) with 10% Fetal Bovine Serum (FBS, Gibco). Hep2 cells were incubated in a humidified incubator with 37°C temperature and 5% CO₂.

Animal experiments

Animals were obtained from the Institute of Zoology, Chinese Academy of Sciences. Animal experiments were performed on basis of the guidelines for the care and utilization of experimental animals of the Institute of experimental animals. The following study protocol has been approved by the IRB of the medical center. Animal care and treatment were conducted on basis of the guidelines for the care and utilization of experimental animals of the NIH. At the end of the experiment, the animals were killed by intraperitoneal injection of excess pentobarbital sodium (4%, 200 mg/kg; Sigma, Shanghai, China). The lung of each group was then collected for further analyses.

Co-immunoprecipitation assay

Co-IP assay was performed following the manufacturer's instructions (Thermo Scientific)³.

RNA Extraction and Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then was reverse transcribed to cDNA using PrimeScript Reverse Transcriptase system (Promega, Madison, WI, USA). QRT-PCR was performed to quantify IL-6 mRNA level with the SYBR Green PCR core Reagent kit (Applied Biosystems, Foster city, CA, USA). GAPDH was used as the endogenous reference. Data were analyzed by using the comparative Ct method. Specificity of resulting PCR products was confirmed by

melting curves. The primers were designed using Primer Express v 2.0 software (Applied Biosystems, Foster City, CA, USA). The primers used in this assay were: ALG3: forward, 5'-CACCTTCTGGGTCATTCACAGG-3' and reverse, 5'-GTGTCACCCTGCAGTTGGGTATAGT-3'; GAPDH: forward, 5'-CTCCTCCACCTTTGACGCTG-3' and reverse, 5'-TCCTCTTGTG CTCTTGCTGG-3'.

Western blot analysis

TU686 and NH8 cells were treated with the treatment of AURKA inhibitor (VX680) for corresponding time. Cells were lysed with RIPA buffer (Pierce, Rockford, USA) to extract the protein (The inhibitor was preserved by Selleck Chemicals company, Houston, TX, USA). And the concentration was measured with the BCA Protein Assay Kit. Proteins with an equal amount (100 µg/sample) were electrophoresed by 10% SDS-PAGE for 2 h and transferred onto 0.22 µm PVDF membranes (Millipore, MA, USA). Membranes were incubated with primary antibodies (anti-p-AURKA (1:2000, Cell Signaling Technology), anti-p-ALG3 (1:2000, Cell Signaling Technology) and GAPDH (1:5000, Abcam).) overnight at 4°C. After membranes were incubated with secondary antibody (1:5000, Cell Signaling Technology) for 2 h, the proteins were visualized using enhanced chemiluminescence detection system (Bioscience, Piscataway, NJ, USA).

CCK8 assay and Plate colony formation assay Cells

96-well plates seeded 2×10^3 cells in 100 µl of DMEM. CCK8 (10 µl) was added to every well. Cells were incubated for 2 h and OD450 absorbance values were measured. Cells were seeded into 6-well plates at 1×10^3 and 2×10^3 cells/well and cultured for 3 weeks with DMEM. Then cells were washed twice with PBS and stained with crystal violet for 30 min. Finally, cell colonies in every well were counted.

In Vitro cell migration and invasion assays

2×10^5 treated cells after overnight starvation were plated in the coated filters in 100 µl of serum-free medium. And 600 µl of medium containing 10% FBS was added to the lower chamber. The insert chambers' membrane was coated by Diluted Matrigel (BD Biosciences) for measuring the cells invasion. All cells were counted under a high-power objective (10x) in random fields. For migration assays, the upper chamber membranes were plated on top of uncoated (Matrigel-free) filters.

In vivo metastasis

4-week-old male immunodeficient mice maintained by the animal resources facility of the medical school of Shanghai Jiaotong University. Animal care and experiments are performed following the "guidelines for the care and utilization of experimental animals" and "principles for the utilization and care of vertebrates", and are approved by the ethics committee of experimental animals of the Medical College of Shanghai Jiaotong University. The average volume and bodyweight of the four groups of mice were similar. The experimental animals were grouped according to the randomization formula. Researchers were not aware of the group allocation at the different stages of the experiment during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis. Mice was randomly assigned

to 5 groups (five in each group). Cells were inoculated via tail vein into the mice. After six months, counting pulmonary metastatic nodules by H&E staining.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. The relationship between the ERp19 expression level and clinicopathologic parameters were calculated with the Pearson χ^2 test. Significant differences between groups were determined using the student t test. Survival data analysis was performed using the Kaplan–Meier and log-rank tests (GraphPad Prism software v6.0).

Declarations

Approval and consent

This study was approved by Institutional Ethnic Committee.

Consent for publication

All authors are consent for publication.

Availability of data and material

The data and material during the current study were available from the corresponding author on reasonable request.

Competing interests

There is on competing interests.

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Author contribution statemen

Performed experiments: YLY,Data analysis: YLY,Manuscript writing: YLY, Revised the manuscript: AH, Study design: AH,HSX, Data interpretation: PPQ, AH

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Not Applicable.

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Tables

Table 1. Relationship between ALG3 expression level and clinicopathological characteristics in 60 LSCC patients.

clinicopathological characteristics	ALG3 staining		P
	Weak	Strong	
Age(years)			
≤60	11	16	0.223
>60	21	12	
Gender			
Male	22	14	0.874
Female	15	9	
tumor size (cm)			
≤5	16	20	0.037
>5	15	9	
invasion range			
T1, T2	9	15	0.014
T3, T4	21	15	
lymph node involvement			
No	8	13	0.032
Yes	23	16	
TNM stage			
I+II	6	18	0.001
III+IV	26	10	

Figures

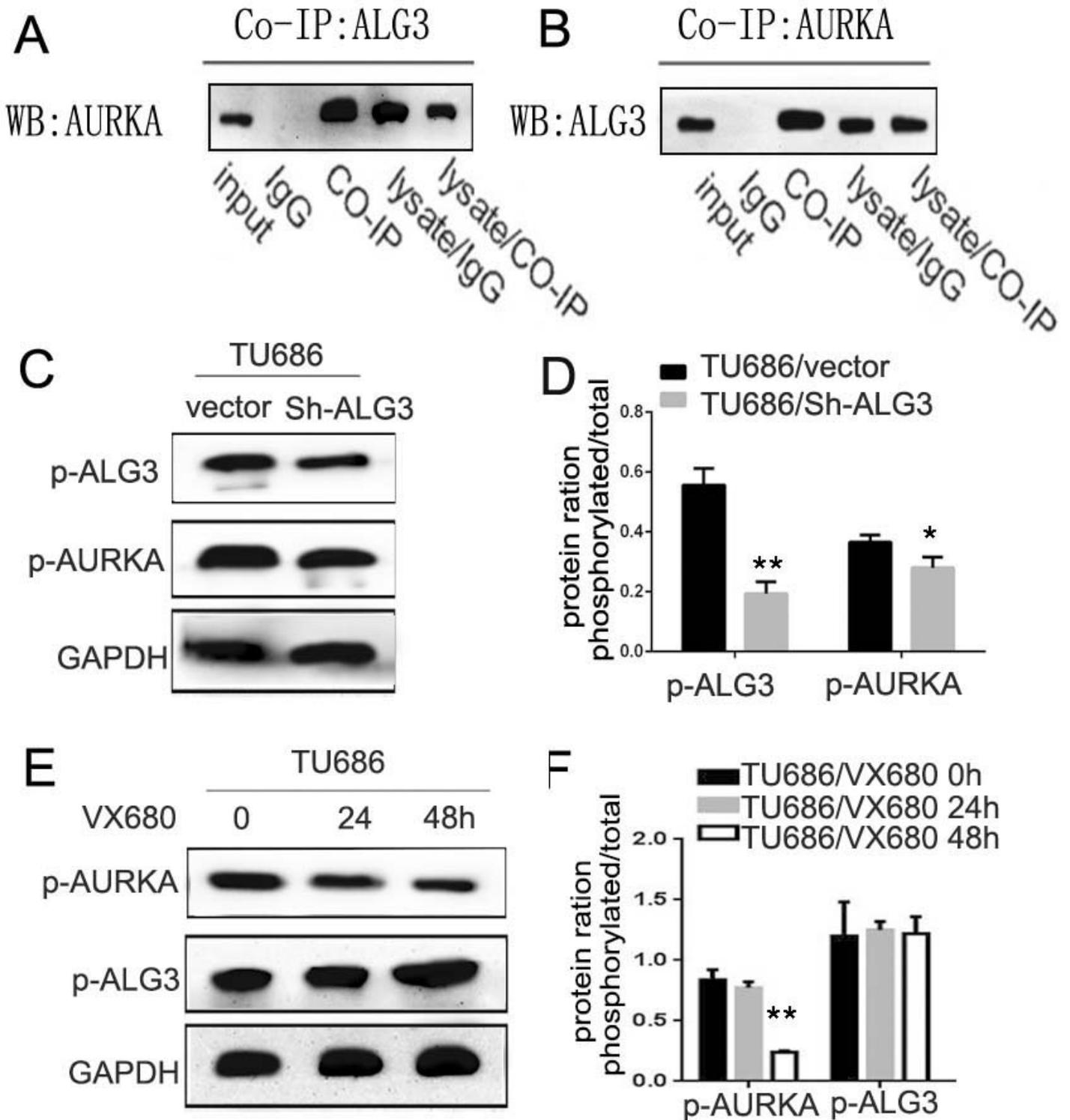


Figure 1

ALG3 corelated with AURKA, ALG3 was the upstream molecular of AURKA. A.B. Co-IP assay was applied to explored the relationship between ALG3 and AURKA. C. Plasmid construction and transfection were applied in down-regulating the expression of ALG3. D. Protein ratio of p-ALG3 and p-AURKA in TU686 cells

(*P<0.05, **P<0.01). E. VX680 was applied in down-regulating the expression of AURKA. F. Protein ratio of p-ALG3 and p-AURKA in TU686 cells (**P<0.01).

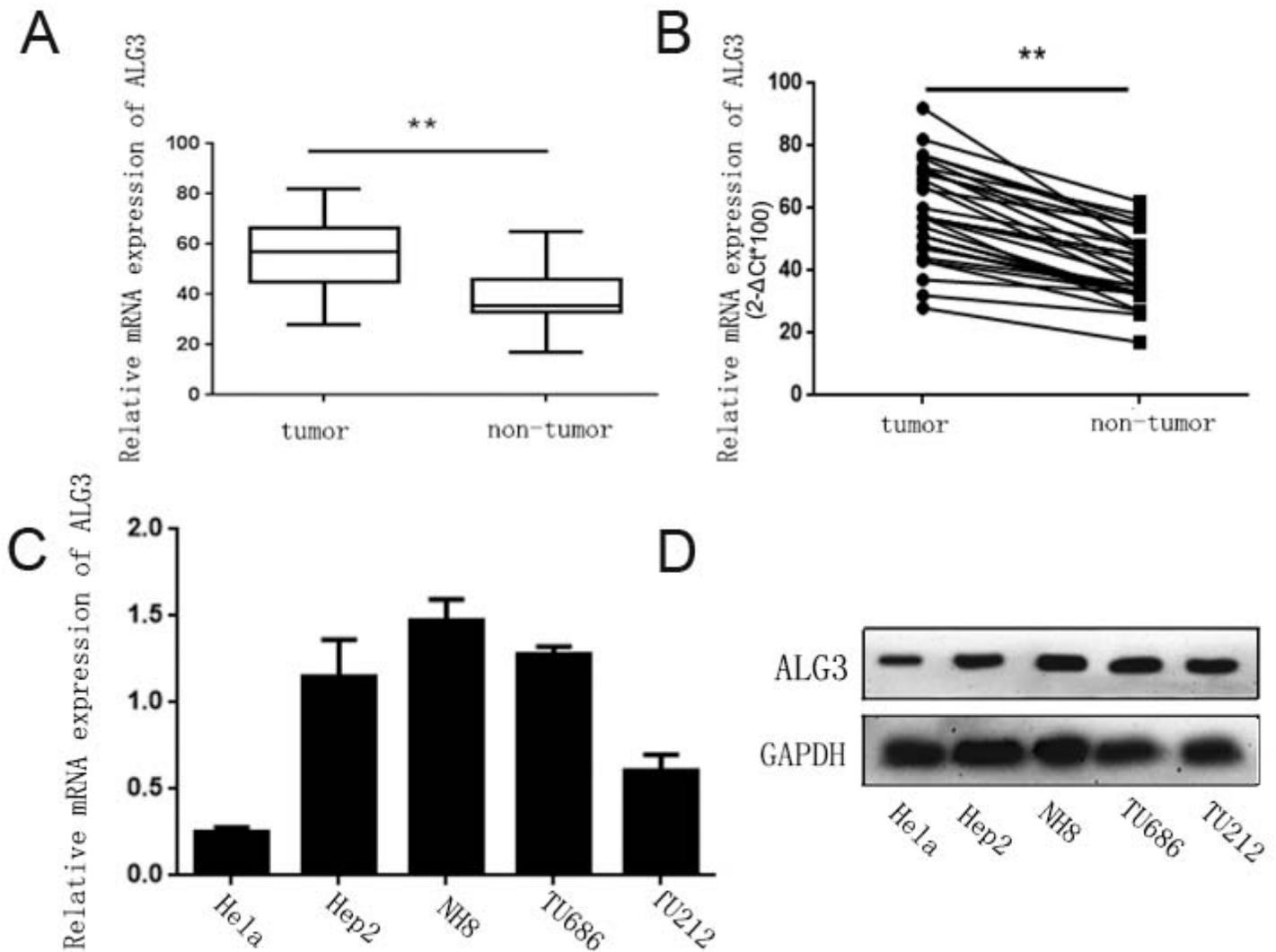


Figure 2

ALG3 expressions in LSCC tissues and cell lines. A.B. qRT-PCR was used to analyze relative mRNA expression of ALG3 in 30 LSCC tissues by qRT-PCR. Data is shown as 2- Δ Ct (** P<0.01). C.D. qRT-PCR and western blotting were respectively used to analyze relative mRNA and protein expression level of ALG3 in LSCC cell lines (Hep2, NH8, TU686, TU212) and cell line (Hela).

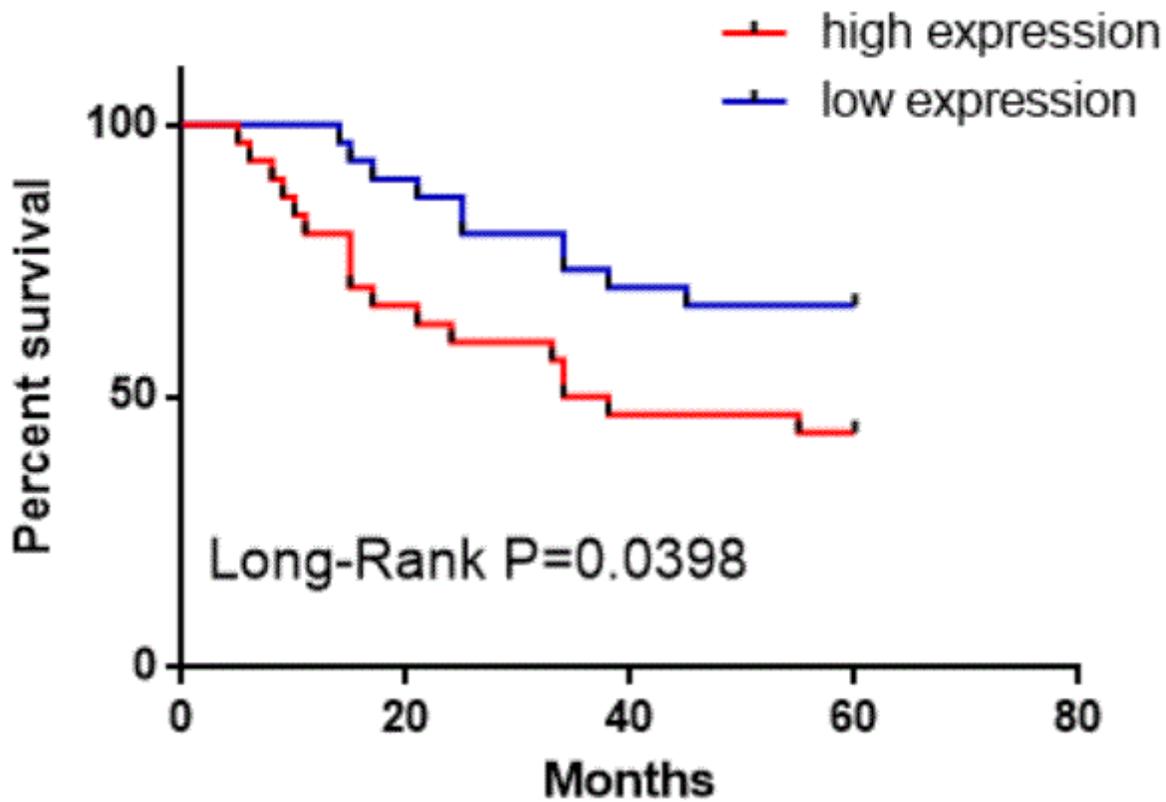


Figure 3

Kaplan-Meier survival curves in LSCC on the basis of ALG3 staining. Patients with ALG3 weak staining had a significantly benign prognosis than those with strong staining, $P=0.0398$.

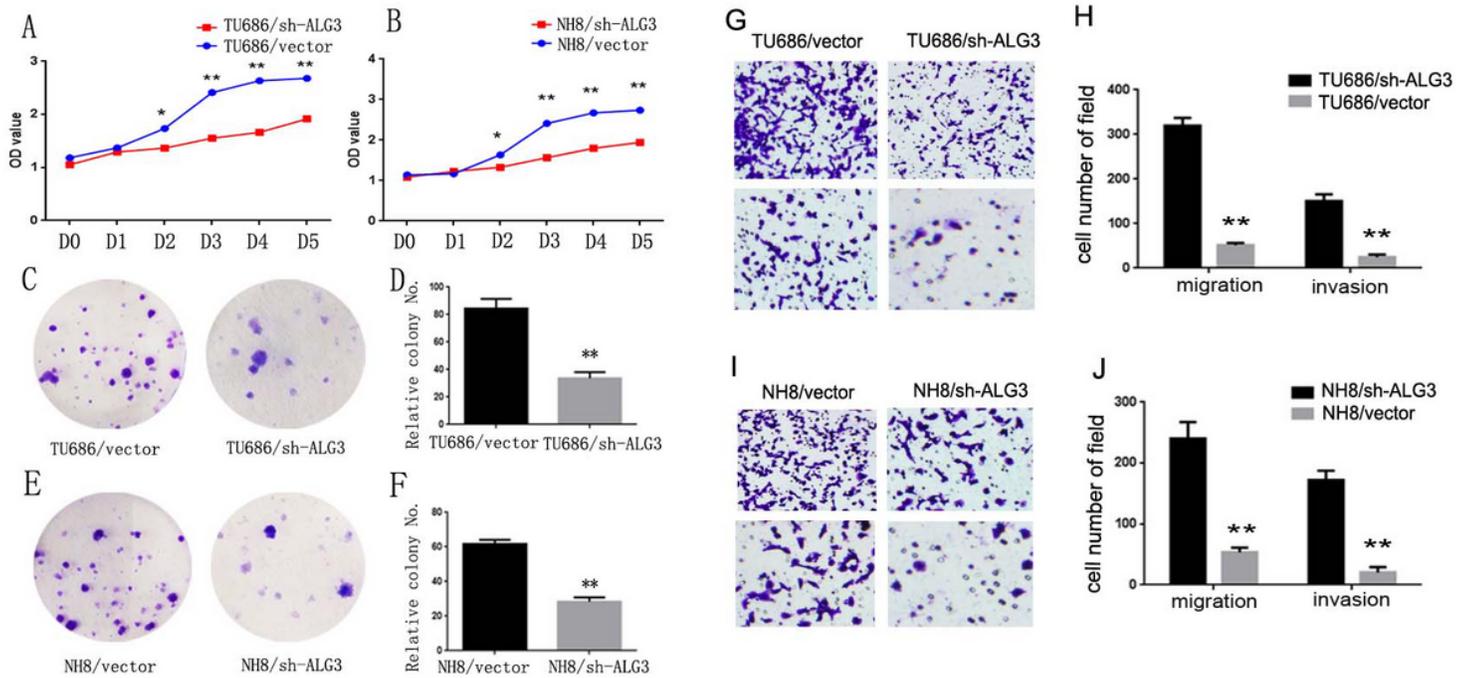


Figure 4

ALG3 promoted LSCC cellular ability of proliferation migration and invasion. **A.B.** Colony formation assays were used to analyze the ability of proliferation of TU686/sh-ALG3, NH8/sh-ALG3 cells, TU686/vector and NH8/vector cells (* $P < 0.05$, ** $P < 0.01$). **C.E.** Colony formation assays were used to explore the ability of proliferation of TU686/sh-ALG3, NH8/sh-ALG3 cells, TU686/vector and NH8/vector cells. **D.F.** Relative colony number of those cells (** $P < 0.01$). **G.I.** Cell migration and invasion assays were performed to evaluate the ability of migration and invasion of TU686/sh-ALG3, NH8/sh-ALG3 cells, TU686/vector and NH8/vector cells. **H.J.** Cell number of fields in those cells (** $P < 0.01$).

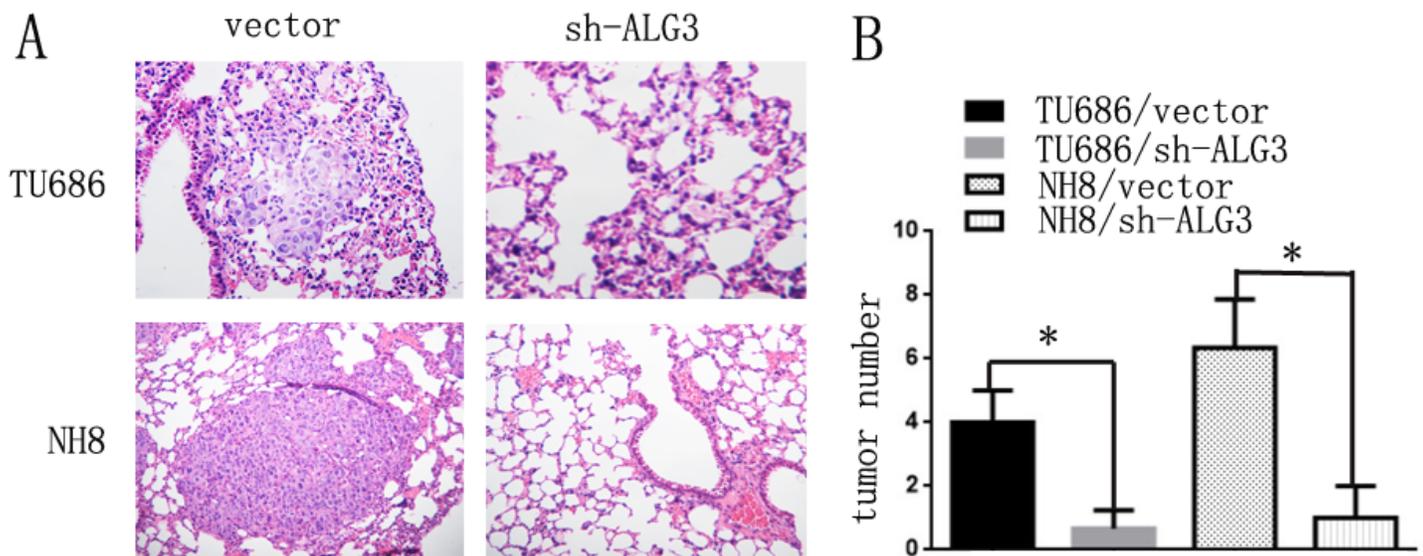


Figure 5

Blocking ALG3 impaired the metastasis of LSCC in vivo. **A.** TU686/vector cells, NH8/vector cells, TU686/sh-ALG3 cells and NH8/sh-ALG3 cells were inoculated into nude mice and pulmonary nodules were observed after six weeks (N=5/group), H&E stains of pulmonary nodules (100×), TU686/vector cells and NH8/vector cells verified larger and more frequently lung metastases with respect to TU686/sh-ALG3 cells and NH8/sh-ALG3 cells. **B.** Pulmonary tissue and nodules were quantified by H&E staining (*P<0.05).