

ALG3 Contributes to the Malignant Biological Properties of Oral Squamous Cell Carcinoma Cells Through Regulating CDK-Cyclin Pathway

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Abstract

Background: In this study, we planned to investigate the function and potential mechanisms of Alpha-1,3-mannosyltransferase (ALG3) in oral squamous cell carcinoma (OSCC).

Methods: Data from The Cancer Genome Atlas (TCGA) was used to analyze ALG3 expression and its effect on the prognosis of patients with OSCC. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was applied to explore the signaling pathways related to ALG3. In OSCC cells, ALG3 expression was measured by qPCR and western blot. Cell counting kit-8, colony formation, and transwell assays were implemented to detect the effects of ALG3 on the malignant biological properties OSCC cells. The expression of key proteins related to CDK-Cyclin pathway was detected by western blot.

Results: The expression of ALG3 in OSCC samples was higher than that of the control samples, and the increase of ALG3 expression was related to unfavorable prognosis of OSCC patients. Additionally, the elevated expression of ALG3 was associated with pathological stage, lymph node metastasis and primary lesion in OSCC patients. ALG3 depletion blocked the growth, colony formation, invasion and migration of OSCC cells, while over-expression ALG3 reversed these phenomena. Moreover, exhaustion of ALG3 resulted in decreased expression of MCM7, CCNB2, CDK1 and PCNA, while these phenomena were inversed after ALG3 up-regulation.

Conclusions: The enhancement of ALG3 expression promoted the aggressive biological behaviors of OSCC cells probably by promoting CDK-Cyclin pathway.

Background

With etiologies primarily referring to the use of tobacco and consumption of alcohol, oral squamous cell carcinoma (OSCC) is one of the most frequently observed neoplasias [1]. More than 500,000 individuals are diagnosed with OSCC each year in the world [2]. Despite the relatively lower metastatic rate (20%), OSCC grows rapidly and typically invades nearby bone and tissue, in addition to owning an extremely high frequency of local recurrence [3]. The major treatments for early OSCC includes surgery and radiation therapy alone or accompanied with chemotherapy [4]. Although considerable progress has been made in the therapeutic of OSCC, the treatment effect and survival rate of OSCC patients are still not satisfied.

Differential expression of glycosyltransferases in cancer cells is one of the dominant reasons of abnormal glycosylation in tumor [5]. While, ALG family represents a crucial class of glycosyltransferases [6]. Alpha-1,3-mannosyltransferase (ALG3) was implicated in the build-up of dolichol linked high mannose type glycans in the endoplasmic reticulum [7]. An increase of ALG3 expression has been presented in multi-drug resistance (MDR) cell lines and peripheral blood mononuclear cells of MDR patients [6]. In steps with previous study, ALG3 expression was significantly up-regulated in esophageal squamous cell carcinoma, primarily in patients with lymph node metastasis [8]. Moreover, up-regulation of ALG3 was obviously related to cervical cancer [9]. Additionally, elevated expression of ALG3 was

associated with the bad overall survival in acute myeloid leukemia patients [6]. Based on the above findings and analysis from ATCC, we speculated that ALG3 was an oncogene in OSCC. However, the function of ALG3 in OSCC still needs to be explored in detail.

This study reported the effect and underlying mechanisms of ALG3 in OSCC for the first time. We obtained that ALG3 was significantly over-expressed in OSCC samples, and its over-expression was positively correlated with OSCC patients worse prognosis. Moreover, the increase of ALG3 expression was related to pathological stage, lymph node and primary lesion in patients with OSCC. Besides, the *in vitro* experiments revealed that over-expression of ALG3 was positively implicated in the regulation of malignant behaviors of OSCC cells. The mechanism of ALG3 over-expression in OSCC cells growth and aggressiveness was mediated by CDK-Cyclin pathway. Our observations might afford a novel molecular for OSCC diagnosis and therapy.

Methods

Bioinformatics analysis

The Cancer Genome Atlas (TCGA, <http://gdc.cancer.gov/>) database including 338 OSCC tumor tissues and 32 normal tissues was used to detect ALG3 expression in OSCC, and evaluate the relationship between ALG3 expression and patients overall survival. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed to detect the pathway closely related to ALG3 in OSCC. Clinical correlation analysis was implemented to assess the association between ALG3 expression and clinical parameters.

Cell culture and gene expression

Cell Bank of the Chinese Academy of Sciences (Shanghai, China) provided the cell lines including OSCC cell lines (CAL27 and TCA-83) and normal cell line HOEC. All cells were sustained in Roswell Park Memorial Institute (RPMI) 1640 medium, which accompanied by 10% fetal bovine serum (FBS), 100U/mL penicillin, and 0.1 mg/mL streptomycin, under an atmosphere including 5% CO₂ at 37 °C.

ALG3 over-expression or knockdown assays were carried out by using pcDNA3.1-ALG3 and si-ALG3. Sequences of si-ALG3 and si-negative control (NC) were exhibited as follows: si-NC: 5'-UUCUUCGAAGGUGUCACGUTT-3', si-ALG3#1: 5'-GGUUUCGUGUACAUCUUUAUG-3', si-ALG3#2: 5'-GGACCUGAGUCUACCCUCAGG-3'. Si-NC, si-ALG3#1, si-ALG3#2, pcDNA3.1-vector, pcDNA3.1-ALG3 were brought from Sangon (Shanghai, China). When the confluence of cells in the six-well plates reached 80%, Lipofectamine2000 was applied to mediate cell transfection. After cultivation for 24 h, the expression of ALG3 could be observed.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Trizol reagent was applied to isolate the total RNA according to the supplier's recommendations. Equal amount of RNA was utilized to synthesize cDNA by PrimeScriptTMRT reagent Kit (Takara, Dalian, China). cDNA abundance was detected by qRT-PCR with SYBR Premix EX Taq (Takara, Dalian, China). $2^{-\Delta\Delta C_t}$ method was used to determine the relative expression of ALG3. GAPDH was regarded as an internal standard for ALG3 detection. The following primers were used:

ALG3: F-5'-CTTTGCTGTGCTCTACCTGGCT-3',

R: 5'-CGCAGCACAAAGATGGAGTGGA-3';

GAPDH: F: 5'-GTCTCCTCTGACTTCAACAGCG-3',

R: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Western blotting

Proteins samples from CAL27 and TCA-83 cells were lysed in RIPA buffer (Beyotime, Shanghai, China) on ice. A total of 20 μ g proteins were isolated on 12% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto membranes. After blocking with 5% non-fat milk for 2 h at ambient temperature, the membranes were incubated with the primary antibodies against the target proteins (Abcam, Cambridge, UK) including ALG3, MCM7, CCNB2, CDK1, PCNA and GAPDH at 4 °C overnight. Thereafter, the membranes were rinsed with tris buffered saline tween (TBST) for three times and then incubated with indicated secondary antibodies. Finally, chemiluminescence analysis was implemented to analyze the signals with the support of the enhanced chemiluminescence (ECL).

Cell proliferation examination

Cell proliferation was evaluated with the assistance of cell counting kit-8 (CCK-8, Beyotime, Shanghai, China) in steps with the supplier's specification. Firstly, a total of 1000 CAL27 or TCA-83 cells were implanted in triplicate into 96-well plates and cultured in 100 μ L RPMI-1640 medium. The optical density (OD) values of the cells were measured every 24 h. Before the measurement, 10 μ L CCK8 solution was put into the medium and incubated for 2 h. The OD values were measured under the absorbance of 450 nm by using a microplate reader. Finally, a growth curve was made by using Graphpad Prism 6.0.

Plate clone formation assay

Firstly, 3mL of complete medium including 200 cells was added into a well from a 6-well plate and cultured at 37 °C and 5% CO₂ incubator for about 10-14 days. Afterwards, cells were lightly rinsed and stained. Finally, the colonies were counted and pictured.

Detection of cell invasion and migration abilities

Transwell chambers (8 µm pore size, Corning Costar, Cambridge) were used to detect the invasion and migration abilities of CAL27 or TCA-83 cells. For invasion experiment, 1×10^5 cells suspended in 200 µL serum-free RPMI-1640 medium were seeded in triplicate into the upper chamber of 24-well chambers. The under compartment included 500 µL RPMI-1640 medium with 10% FBS. After cultivation in 37 °C incubator for 24 h, the chambers were rinsed by phosphate buffer solution (PBS) gently to remove the cells gluing to the upper surface. After being fixed and stained, the cells were photographed under a microscope.

The procedure of the migration experiment was consistent with that of the invasion experiment, except that the upper chamber did not need to be pre-coated.

Data analysis

The values in this research were exhibited as mean \pm standard deviation (SD). Statistical analysis was implemented by SPSS22.0 and Graphpad Prism 6.0. The comparisons between the experimental group and control group were evaluated by student's t test and one way ANOVA. The overall survival of OSCC patients with different expression of ALG3 was analyzed by Kaplan-Meier method with the log-rank test. Chi-square analysis was used to assess the association between the ALG3 expression and the clinicopathological parameter of OSCC. A P value greater than 0.05 was considered statistically insignificant.

Results

ALG3 expression was associated with overall survival of OSCC patients and cell cycle/replication related pathways

By analyzing the TCGA database including 338 OSCC tumor and 32 normal tissues, ALG3 was picked up for further exploration. The data from TCGA presented that ALG3 was obviously enhanced in OSCC tumor tissues, which was consisted with the expression in esophageal squamous cell carcinoma and breast cancer [8,10](**Fig. 1A**, $p=4.735e-03$). In addition, high expression of ALG3 was related to pathologic stage, lymph node metastasis, and primary lesion in patients with OSCC (**Table 1**). The prognostic value of ALG3 expression for patients with OSCC was analyzed by Kaplan-Meier plotter. As exhibited in **Fig. 1B**, ALG3 high expression in OSCC was significantly related to shorter overall survival. Thereafter, KEGG pathway analysis was performed to study the role of ALG3 and potential underlying mechanisms of OSCC. The data from **Fig. 1C-D** showed that ALG3 expression was closely related to cell cycle and DNA replication pathways. The above findings provided a basis for our following experiments.

Detection of ALG3 expression in different condition

To elucidate the impact of ALG3 on OSCC cells biological characteristics, we first detected ALG3 expression. The data from **Fig. 2A** exhibited that an increased expression of ALG3 was appeared in CAL27 and TCA-83 cells. Afterwards, si-ALG3 and pcDNA3.1-ALG3 were used to intervene ALG3

expression. The data in **Fig. 2B-D** presented that ALG3 mRNA and protein expression levels were profoundly elevated after pcDNA3.1-ALG3 stimulation. While, after si-ALG3#1 or si-ALG3#2 treatment, ALG3 expression was obviously decreased, especially in si-ALG3#1 group (**Fig. 2E-G**, $p < 0.01$). So, si-ALG3#1 and pcDNA3.1-ALG3 were selected for the subsequent experiments.

ALG3 played a positive role in OSCC cells growth and aggressiveness

To investigate the function of ALG3 on CAL27 and TCA-83 cells malignant biological properties, CCK-8, colony formation, and transwell assays were implemented. After up-regulation of ALG3, the OD values, the number of clones, invasion and migration of CAL27 cells increased significantly (**Fig. 3A, C, E**, $p < 0.01$). While, in TCA-83 cells, depletion of ALG3 blocked the growth ability as we observed an obvious decrease of OD values and colony number (**Fig. 3B, D**, $p < 0.01$). Moreover, the number of invaded and migrated TCA-83 cells were significantly decreased after ALG3 knockdown (**Fig. 3F**, $p < 0.01$). These discoveries illustrated that ALG3 promoted malignant behaviors in OSCC cells by elevating cells proliferation and metastasis.

Cell cycle and replication related genes were regulated by ALG3

From KEGG enrichment analysis, ALG3 was discovered to be related to cell cycle and DNA replication. It is well documented that cyclin dependant kinase 1 (CDK1), Minichromosome maintenance complex component 7 (MCM7), cyclin B2 (CCNB2), and proliferation cell nuclear antigen (PCNA) participated in cell cycle progression, which has been confirmed in several tumors including OSCC [11-14]. Importantly, in this study, we discovered that the expression of MCM7, CCNB2, CDK1, and PCNA were significantly elevated in CAL27 cells after ALG3 up-regulation. Whilst, in TCA-83 cells, depletion of ALG3 suppressed the expression of MCM7, CCNB2, CDK1, and PCNA. These observations illustrated that the promoting effects of ALG3 on OSCC cells malignant behaviors were carried out partly through regulating MCM7, CCNB2, CDK1 and PCNA expression.

Discussion

In current study, we revealed that ALG3 can promote the development of OSCC. ALG3 was profoundly increased in OSCC tissues, and this increase in ALG3 expression was positively connected with worse prognosis of OSCC patients. Significantly, it was demonstrated that ALG3 played a role as an oncogene in OSCC by enhancing CDK-Cyclin pathway.

According to previous studies, ALG3 has been reported to be an underlying treatment target in non-small cell lung cancer [15], and high expression of ALG3 was positively correlated with the rapid development and poor prognosis of breast cancer patients [10]. Similar to previous studies, our results discovered that ALG3 was highly expressed in OSCC tissues and cell lines. We have presented a significant association of ALG3 expression with the prognosis of OSCC patients. Moreover, over-expressed ALG3 accelerated the malignant behaviors of OSCC cells.

Cellular programs such as proliferation, apoptosis, differentiation, and senescence are intimately related to the cell cycle regulatory machinery [16]. Cell cycle pathway is frequently activated in human tumors, including OSCC [17]. MCM7 performs a crucial function in controlling the progress of the cell cycle and the initiation of DNA replication [18]. For example, several researches have indicated that MCM7 over-expression is related to the presence of certain neoplasms including esophageal [19], gastric [20], or hepatic cancers [21], acute myeloid leukemia [22], and chronic myeloid leukemia [23]. By KEGG enrichment analysis, ALG3 was discovered to be related to DNA replication. Therefore, we examined the relationship between ALG3 and MCM7, which plays an important role in DNA replication. Our results showed that MCM7 expression was obviously up-regulated in ALG3 up-regulation condition, confirming the positively relationship between ALG3 and MCM7.

It is well documented that different cyclins modulate the eukaryotic cell cycle in response to CDKs at specific points of the mitotic cycle [24]. And cell cycle development follows periodic alternations in the protein levels of cyclins, A, B, D and E [24]. Moreover, a previous study has reported that cyclin B2 (CCNB2) induces G2/M transition by activating CDK1 kinase, and CCNB2 suppression contributes to cell cycle arrest [25]. Significantly, analysis from KEGG enrichment revealed that ALG3 expression was related to cell cycle pathway. More importantly, our study discovered that CDK1 and CCNB2 were significantly up-regulated in ALG3 over-expression condition. It is noted that CCNB2 and CDK1 were significantly up-regulated in the OSCC tumors compared with the normal [12]. Furthermore, compared with previous study, we discovered that ALG3 can promote malignant behaviors of OSCC cells by up-regulating CCNB2 and CDK1.

PCNA, as a nuclear protein and marker of cell proliferation, is discovered to be powerfully associated with prognosis and survival in multiple types of solid malignancies, such as colorectal cancer [26], breast cancer [27] and OSCC [28]. In OSCC, previous studies revealed that PCNA expression presented a positive association with histological grading [29], with an increase in PCNA expression being related to a poorly differentiated tumor and a decreased expression of PCNA suggestive of well-differentiated OSCC [14]. Significantly, our study discovered that PCNA expression was obviously increased after ALG3 over-expression, indicating that ALG3 might promote malignant progression of OSCC cells by upregulating PCNA.

In spite of this, several shortcomings in the study should be pointed out. Firstly, our results need to be validated *in vivo*. Secondly, it is necessary to verify whether the regulation of ALG3 on MCM7, CCNB2, CDK1 and PCNA is direct or indirect. Thirdly, what is the effect of ALG3 co-expression with the above genes on the survival of patients with OSCC.

Conclusion

In summary, we discovered that ALG3 up-regulation resulted in shorter overall survival in OSCC patients, as well as promoted the growth and aggressiveness of OSCC cells through the regulation of CDK-Cyclin pathway.

List Of Abbreviations

ALG3: Alpha-1,3-mannosyltransferase;

OSCC: oral squamous cell carcinoma;

TCGA: The Cancer Genome Atlas;

KEGG: Kyoto Encyclopedia of Genes and Genomes;

RPMI: Roswell Park Memorial Institute (RPMI);

FBS: fetal bovine serum;

SDS-PAGE: sulfate–polyacrylamide gel electrophoresis;

TBST: tris buffered saline tween;

ECL: enhanced chemilunimescence;

CCK-8: cell counting kit-8;

OD: optical density;

SD: standard deviation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

PS and CW designed the experiments, performed the experiments, analyzed the data and wrote the manuscript. YW conceived the research program and reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Relationship between ALG3 expression and clinicopathological properties of patients with oral squamous cell carcinoma.

| Characteristics | Expression of ALG3 | | P value |
|-------------------------|--------------------|------|---------|
| | Low | High | |
| Age | | | 0.804 |
| <60 | 56 | 58 | |
| ≥60 | 77 | 75 | |
| Gender | | | 0.789 |
| female | 41 | 39 | |
| male | 92 | 94 | |
| Grade | | | 0.874 |
| G1+G2 | 109 | 108 | |
| G3+G4 | 24 | 25 | |
| Pathologic-Stage | | | 0.002* |
| I+II | 40 | 19 | |
| III+IV | 93 | 114 | |
| Pathologic-T | | | 0.033* |
| T1+T2 | 61 | 44 | |
| T3+T4 | 72 | 89 | |
| Pathologic-N | | | 0.048* |
| N0 | 67 | 51 | |
| N1+N2 | 66 | 82 | |

*P<0.05, T: tumor, N: lymph nodes, M: metastasis.

Figures

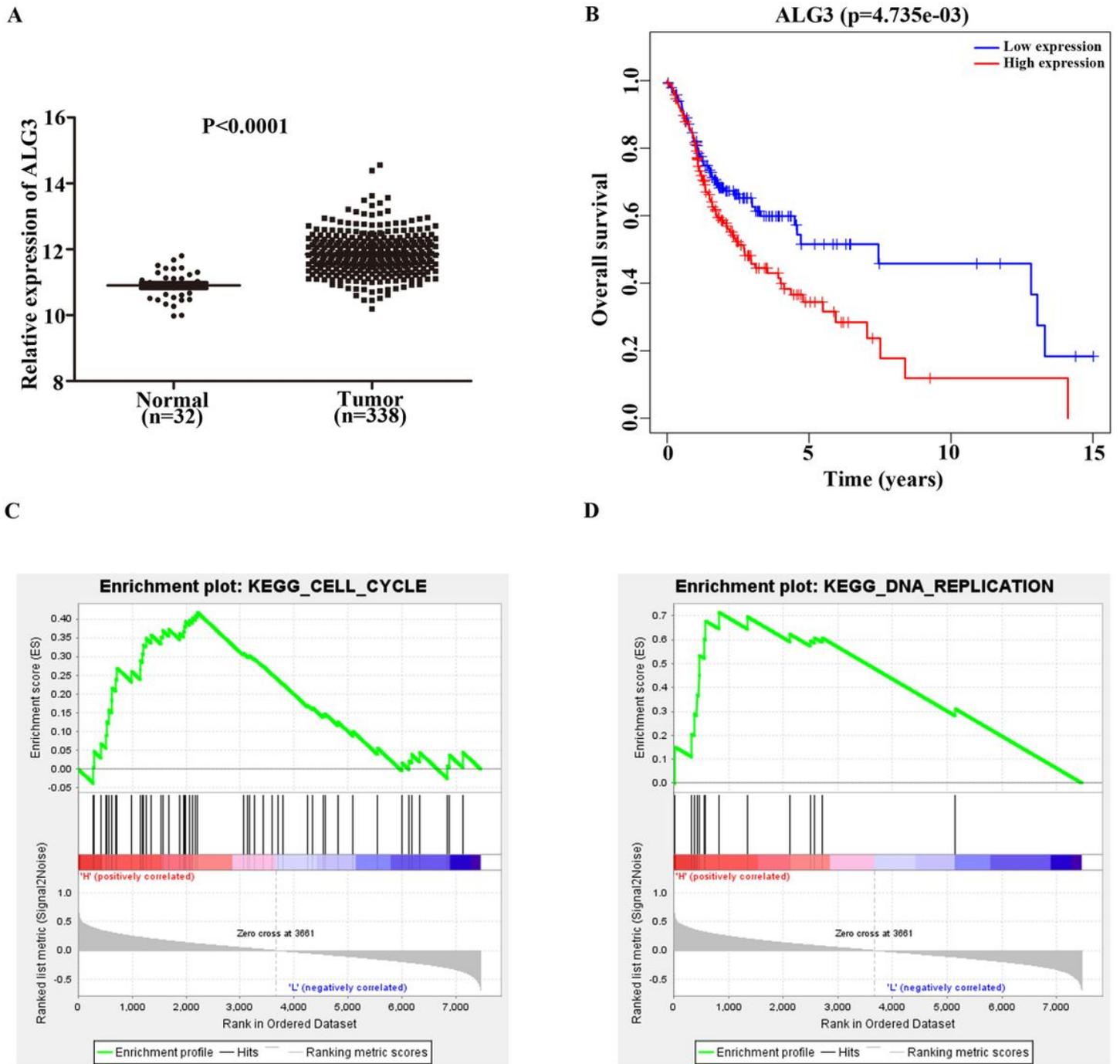


Figure 1

ALG3 was highly expressed in OSCC samples and related overall survival of OSCC patients and cell cycle/DNA replication pathways. A. Data from TCGA including 32 normal and 338 tumor samples showed that ALG3 was highly expressed in OSCC samples, $p < 0.0001$. B. The overall survival of OSCC was shorter in patients with high ALG3 level, $p=4.735e-03$. C-D. Analysis from KEGG showed that ALG3 expression was related to cell cycle and DNA replication pathways.

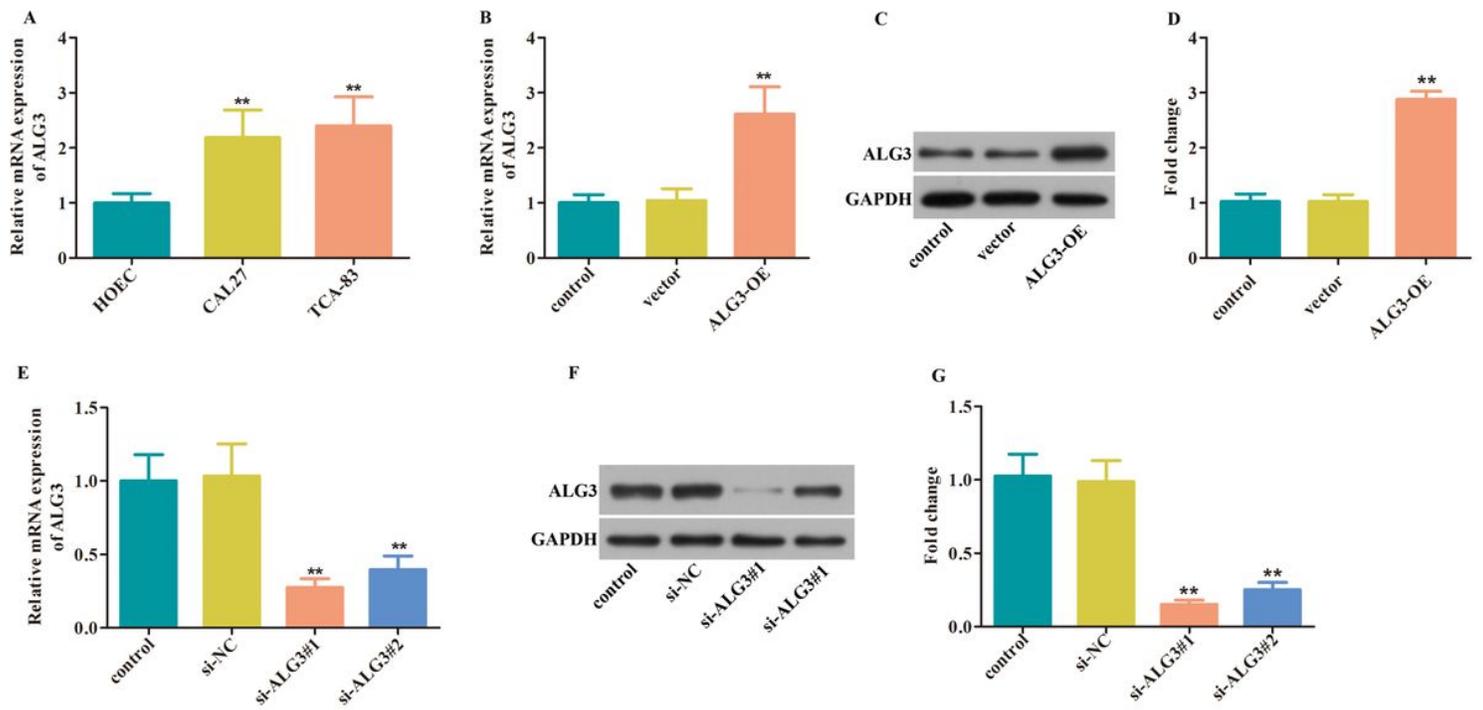


Figure 2

ALG3 expression in OSCC cells was measured by qPCR and western blot. A. ALG3 expression was obviously upregulated in CAL27 and TCA-83 cells compared with HOEC cells, $**p < 0.01$. B-D. The expression of ALG3 in CAL27 cells was measured by qPCR and western blot, $**p < 0.01$. E-G. In TCA-83 cells, the expression of ALG3 was significantly decreased after si-ALG3#1 and si-ALG3#2 treatment, $**p < 0.01$.

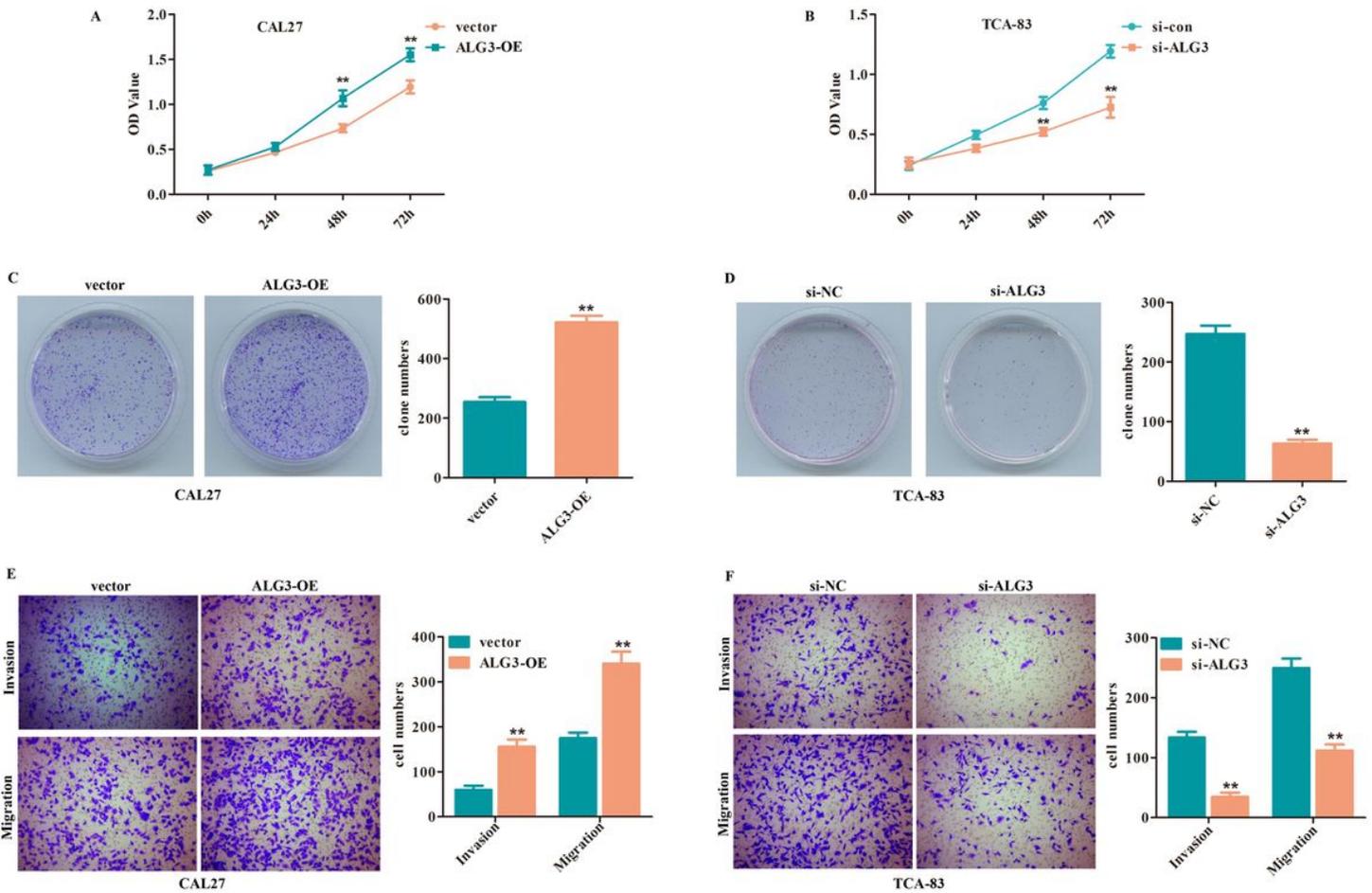


Figure 3

CCK-8, colony formation, and transwell assays were used to detect the effects of ALG3 on OSCC cells. A. The proliferation ability in CAL27 cells was elevated after ALG3 upregulation. B. Depletion of ALG3 decreased TCA-83 cells growth ability. C. Overexpression of ALG3 strengthened the colony formation ability of CAL27 cells. D. The number of clone formation in TCA-83 cells was reduced after ALG3 knockdown. E. The invasion and migration number of CAL27 cells was increased with ALG3 upregulation. F. Exhausting ALG3 attenuated the invasion and migration abilities of TCA-83 cells. ** $p < 0.01$.

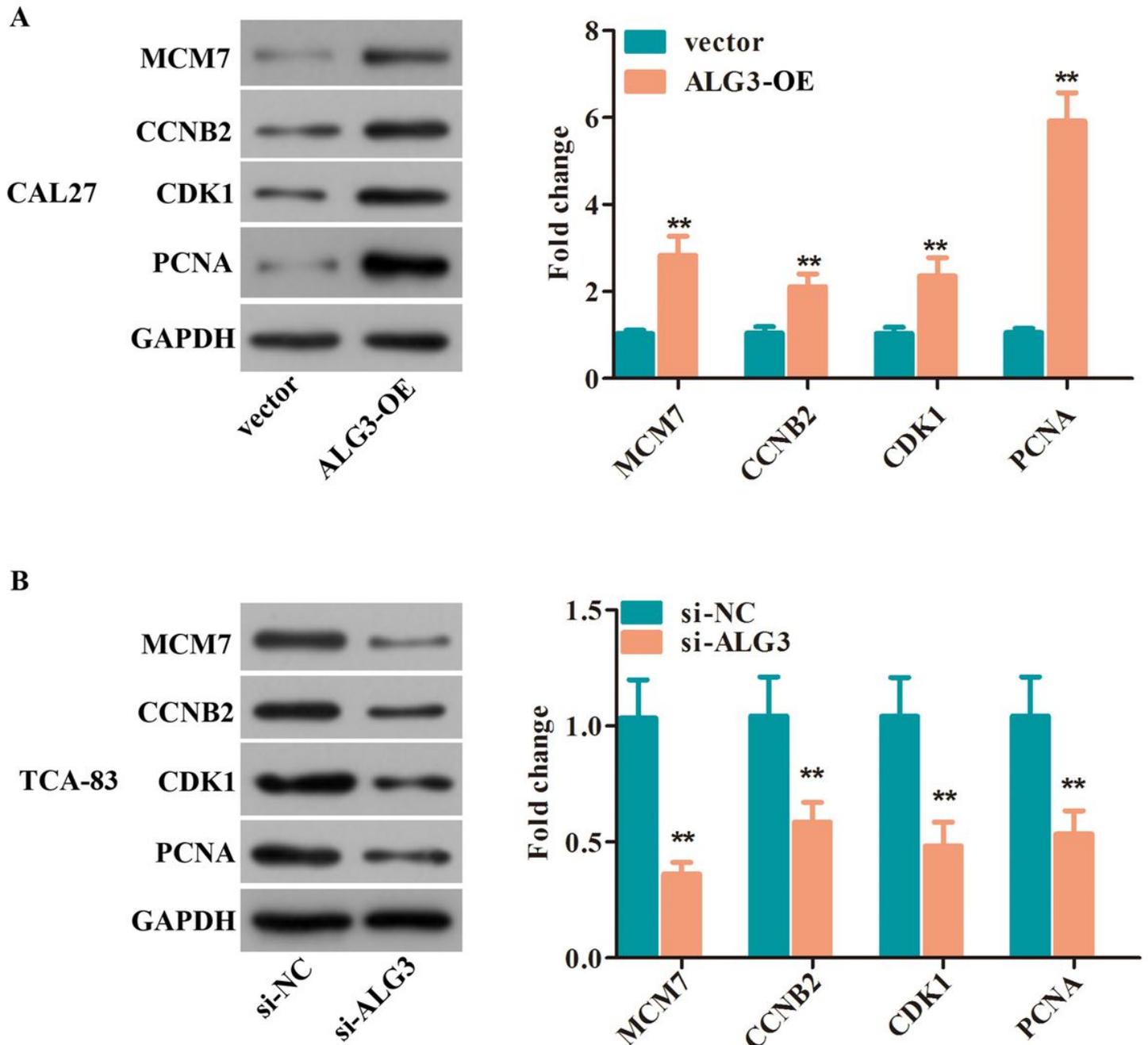


Figure 4

Western blot was used to detect cycle and replication related genes expression. A. The protein expression of MCM7, CCNB2, CDK1 and PCNA in CAL27 cells were elevated after ALG3 overexpression. B. Knockdown of ALG3 inhibited the expression of MCM7, CCNB2, CDK1 and PCNA in TCA-83 cells. ** $p < 0.01$.

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