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Increased LGALS3BP promotes proliferation and migration of oral squamous cell carcinoma via PI3K/AKT pathway

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ABSTRACT

Previous studies showed that lectin galactoside-binding soluble 3 binding protein (LGALS3BP) is an important participant in tumor progression. However, its prognostic value and functional mechanism in oral squamous cell carcinoma (OSCC) are still unclear. In this study, we analyzed LGALS3BP expression in OSCC tissues via Oncomine databases and immunohistochemical staining. LGALS3BP was significantly up-regulated in OSCC tumor tissues. IHC analysis showed that LGALS3BP was predominantly expressed in tumor cells and correlated with poor clinical characteristics. In addition, high LGALS3BP expression predicted poor clinical outcomes and multivariate analysis revealed that LGALS3BP expression was as an independent prognostic factor for OS, DFS and RFS ($p < .0001$, $p = .002$, $p = .002$). Mechanically, LGALS3BP regulated OSCC proliferation and migration via PI3K/AKT pathways, which was abrogated by PI3K inhibitor LY294002 in a dose-dependent manner. Our results suggested that LGALS3BP could be served as a novel independent prognostic factor as well as a potential therapeutic target for OSCC treatment.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant epithelial neoplasm affecting oral cavity and accounts for > 90% of oral cancer. Annually, over 300,000 cases are newly diagnosed, and the morbidity and mortality rates keep rising among young adults in recent years [1]. Despite the recent progress made in radiotherapy, chemotherapy and traditional surgery, the 5-year survival rate of OSCC still hovers around 50% [2]. The prognosis is poor partly due to lack of reliable biomarkers, which limited high-precision medicine. Therefore, in this study we aimed to identify a promising prognostic indicator and explore its biological function in OSCC [3].

Lectin galactoside-binding soluble 3 binding protein (LGALS3BP, also known as 90 K or Mac-2BP) is a member of the scavenger receptor cysteine-rich domain (SRCR) family of proteins [4,5] and significantly elevated in malignant tumors [6,7]. LGALS3BP is reported to be associated with unfavorable clinical outcomes and contributes to cancer progression in multiple ways. High LGALS3BP expression facilitated metastasis by increasing adhesiveness of cancer cells and inhibiting monocyte-derived fibrocyte differentiation [8–10]. Furthermore, LGALS3BP was a novel proangiogenic factor capable of inducing vascular endothelial growth factor (VEGF) expression in human breast cancer cells and promoted angiogenesis by a direct stimulation of endothelial cells [6,11]. In addition, clinical data showed that increased expression of LGALS3BP was associated with a reduced response to chemotherapy in patients with breast carcinoma [10,12], hepatocellular carcinoma [13,14], pleural mesothelioma [15], pancreatic carcinoma [16], non-small cell lung carcinoma [8,17,18] and neuroblastoma [19]. However, LGALS3BP had antitumor effects in colorectal cancer. Both Lee and Enza et al. found that LGALS3BP suppressed colorectal cancer cell growth and high expression of LGALS3BP was associated with better prognosis [20,21]. These studies indicated that the prognostic values and mechanisms of LGALS3BP varied among different tumor types. Thus, the expression of LGALS3BP should be further studied in OSCC.

Although previous studies exhibited that LGALS3BP was elevated in saliva of OSCC patients and its expression was found to be regulated by miR-596 and multiple coagulation factor deficiency protein 2, its prognostic values and biological function in OSCC remain poorly characterized. Here, for the first time, we investigated the expression of

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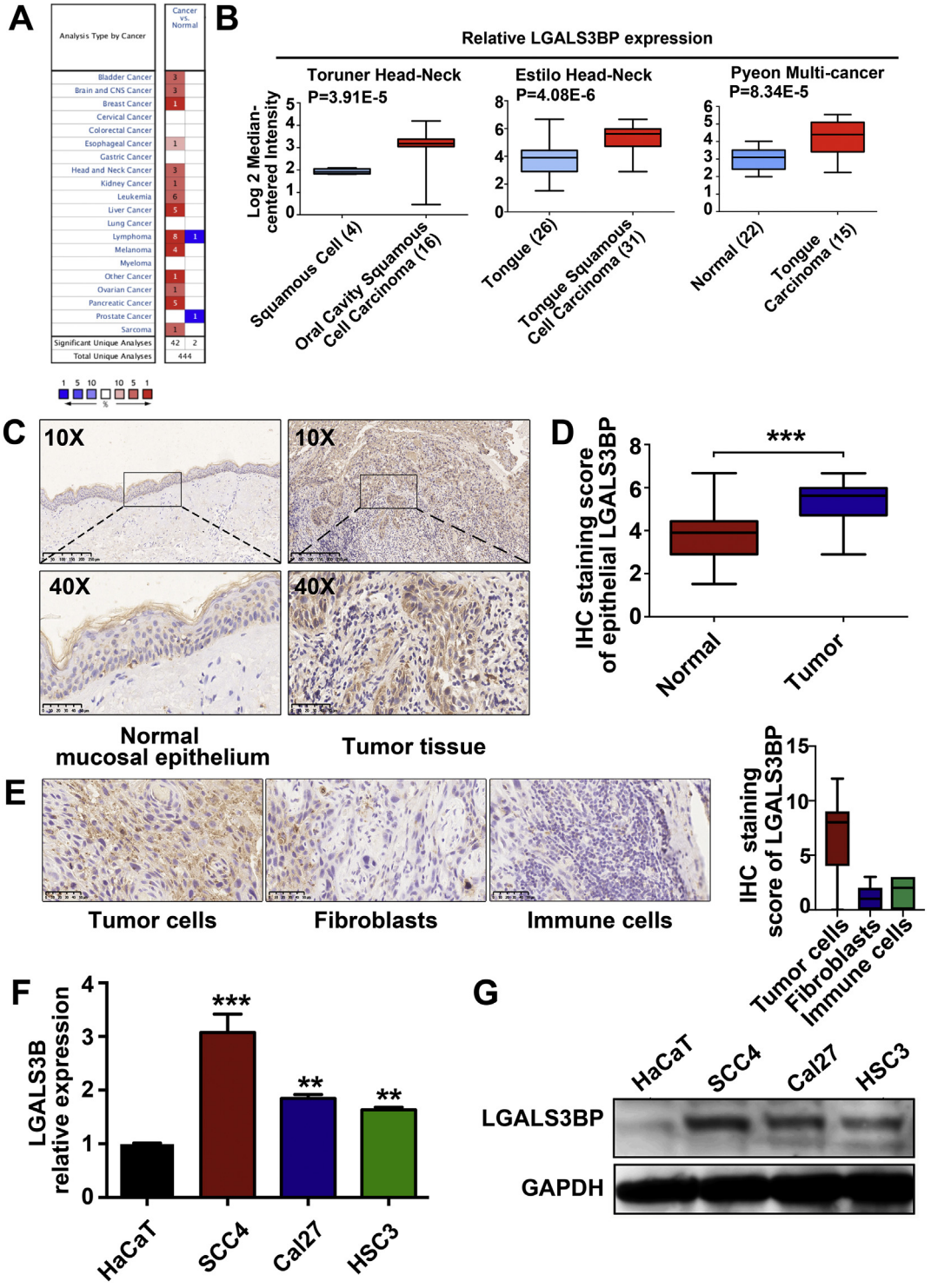

Fig. 1. Expression of LGALS3BP in OSCC.

A) Oncomine analysis of LGALS3BP expression in human cancers (www.oncomine.org). Cell color is determined by the best gene rank percentile for the analyses within the cell; B) LGALS3BP expression levels in human OSCC specimens and the adjacent normal tissues in multiple cancer microarray data sets available from Oncomine; C) Representative images of IHC analysis of LGALS3BP expression between human OSCC specimens and adjacent normal mucosal epithelium; D) Box blot depicting LGALS3BP expression levels by IHC between human OSCC specimens and adjacent normal mucosal epithelium; E) Representative images (left) and box blot analyses (right) of LGALS3BP expression in cancer cells, immune cells and fibroblasts; F) LGALS3BP mRNA levels in three OSCC cell lines and HaCaT cells were analyzed by RT-PCR; G) LGALS3BP expression levels in three OSCC cell lines and HaCaT cells were analyzed by western blots; Data was presented as mean ± S.D.** $p < 0.01$,*** $p < 0.001$.

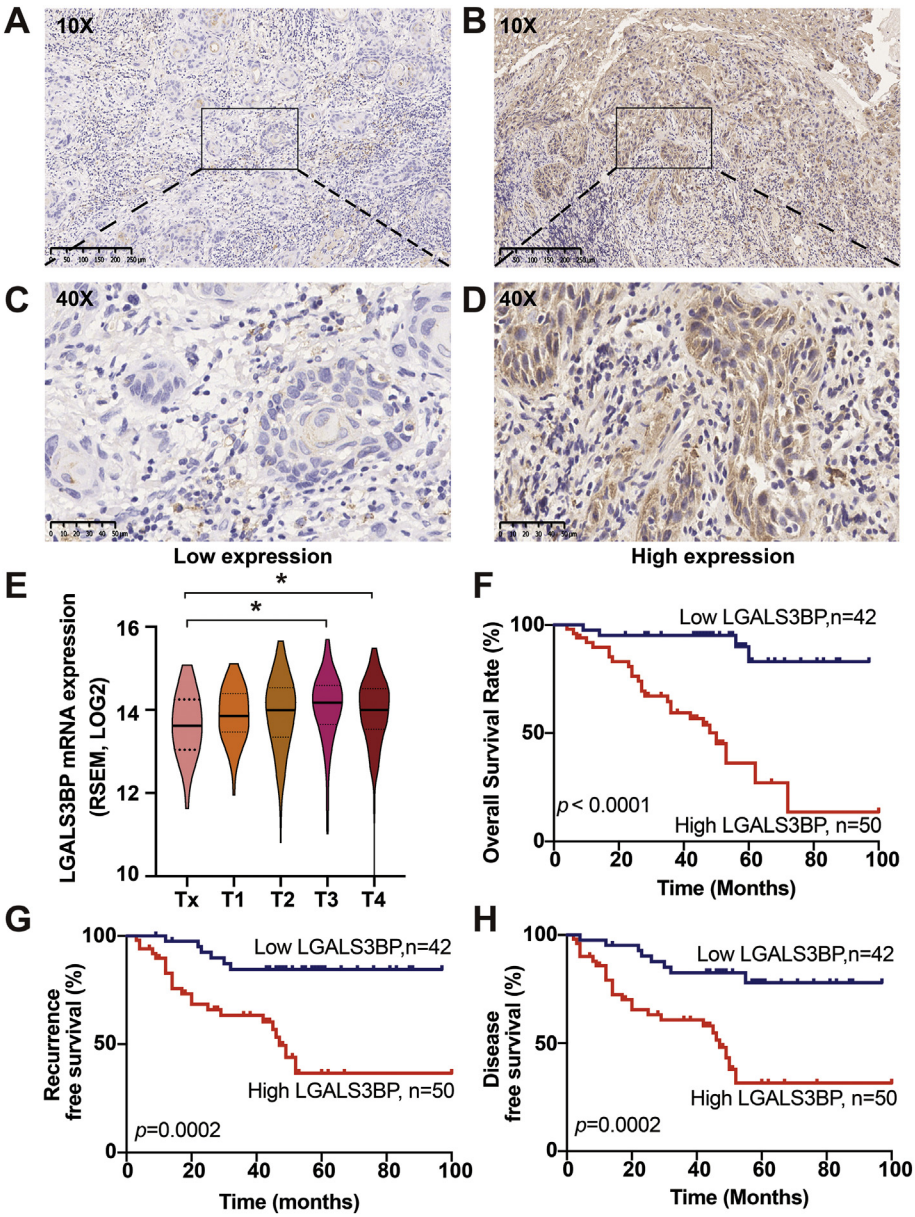

Fig. 2. High expression of LGALS3BP correlated with poor clinical outcomes.
A) IHC staining for LGALS3BP expression with low score (magnification, ×10); B) IHC staining for LGALS3BP expression with high score (magnification, ×10); C) IHC staining for LGALS3BP expression with low score (magnification, ×40); D) IHC staining for LGALS3BP expression with high score (magnification, ×40); E) LGALS3BP expression levels between different T stages of head and neck cancer patients in TCGA database; F) Kaplan-Meier analysis of overall survival for OSCC patients with high LGALS3BP expression versus low LGALS3BP expression; F) Kaplan-Meier analysis of recurrence free survival for OSCC patients with high LGALS3BP expression versus low LGALS3BP expression; G) Kaplan-Meier analysis of disease free survival for OSCC patients with high LGALS3BP expression versus low LGALS3BP expression. * $p < 0.05$.

Table 1
The expression pattern of LGALS3BP in tumor cells.

Intensity	Proportion of positive cells(score)				
Score	0–5% (0)	6%–25% (1)	26%–50% (2)	51%–75% (3)	76%–100% (4)
Weak staining (1)	0 (2/2.17%)	1 (2/2.17%)	2 (3/3.26%)	3 (2/2.17%)	4 (5/5.43%)
Moderate staining (2)	0 (2/2.17%)	2 (2/2.17%)	4 (3/3.26%)	6 (8/8.79%)	8 (18/19.57%)
Strong staining (3)	0 (2/2.17%)	3 (2/2.17%)	6 (7/7.61%)	9 (14/15.22%)	12 (20/21.74%)

LGALS3BP using public datasets and analyzed its diagnostic and prognostic value by immunohistochemistry (IHC) in 92 OSCC patients. Moreover, we investigated the effects and underlying mechanisms of LGALS3BP in OSCC cell lines.

2. Material and methods

2.1. Study population and design

92 OSCC patients treated at the Nanjing Stomatological Hospital, Medical School of Nanjing University between 2008 and 2014 were enrolled in our study. All the patients in this study have not received radiotherapy or chemotherapy before operation. The clinicopathological information of each case, including age, sex, tumor site, tumor size, lymph node metastases, distant metastasis, tumor differentiation, invasion, and perineural invasion were obtained from patient records. The tumor differentiation grade and TNM stage of OSCC were determined by two pathologists according to the eighth edition of the AJCC Cancer Staging Manual. This study was conducted according to the ethical principles of the Helsinki Declaration and approved by the

Table 2
Correlation of LGALS3BP expression in tumor cells and clinicopatholigical characteristics in OSCC patients.

Characteristics	Tumor cells			p
	n = 92 (%)	Low (%)	High (%)	
Gender				
Male	51 (55.4%)	23 (45.1%)	28 (54.9%)	0.905
Female	41 (44.6%)	19 (46.3%)	22 (53.7%)	
Age				
< 60	41 (44.6%)	22 (53.7%)	19 (46.3%)	0.167
≥60	51 (55.4%)	20 (39.2%)	31 (60.8%)	
Smoking				
No	73 (79.3%)	35 (47.9%)	38 (52.1%)	0.387
Yes	19 (20.7%)	7 (36.8%)	12 (63.2%)	
T stage				
I-II	60 (65.2%)	32 (53.3%)	28 (46.7%)	**0.043**
III-IV	32 (34.8%)	10 (31.3%)	22 (68.6%)	
Differentiation				
Well	35(41.8%)	20(57.1%)	15(42.9%)	0.096
Moderate/poor	56(58.2%)	22(39.3%)	34(60.7%)	
WPOI				
1–3	35(38.0%)	20 (57.1%)	15 (42.9%)	0.083
4–5	57(61.9%)	22 (38.6%)	35 (61.4%)	
Nerve invasion				
No	78 (84.8%)	37 (47.4%)	41 (52.6%)	0.418
Yes	14 (15.2%)	5 (35.7%)	9 (64.3%)	
Inflammation				
No	28 (30.4%)	14 (45.1%)	14 (54.9%)	0.580
Yes	64 (69.6%)	28 (43.8%)	36 (56.3%)	
Metastasis				
No	82 (89.1%)	37 (48.6%)	45 (51.4%)	0.770
Yes	10 (10.9%)	5 (50.0%)	5 (50.0%)	
Recurrence				
No	64 (72.0%)	36 (56.3%)	28 (43.7%)	**0.002**
Yes	28 (28.0%)	6 (21.4%)	22 (78.6%)	
Survival				
No	63 (68.5%)	38 (60.3%)	25 (39.7%)	< **0.0001**
Yes	29 (31.5%)	4 (13.8%)	25 (86.2%)	

DOI: depth of invasion
WPOI:worst pattern of invasion

Nanjing Stomatological Hospital ethics committees.

2.2. Database

Oncomine microarray database (http://www.oncomine.org) was used to retrieve the LGALS3BP expression in the OSCC samples. “LGALS3BP” was used as a keyword in the Oncomine search, “Cancer vs. Normal Analysis” was used as the primary filter, and “Head and Neck Squamous Cell Carcinoma vs. Normal Analysis” was chosen as the cancer type. The up-regulated gene levels of LGALS3BP were shown in multiple data sets including Toeuner's, Estilo's and Pyeon's datasets. The LGALS3BP expression data were log-transformed, median-centered per array, and the standard deviation (SD) was normalized to one per array.

2.3. Immunohistochemical (IHC) staining and evaluation

2 μm paraffin-embedded sections of tumor tissues were deparaffinized and treated with 10 mM citrate buffer at 92 °C for 30 min for antigen retrieval. After blocking in 10% bovine serum albumin, the slides were immune-stained with rabbit polyclonal IgG anti-human LGALS3BP (1:200; Proteintech).

The LGALS3BP expression was scored by two independent professional pathologists blinded to the clinical data from 3 randomly selected views for each tumor section under 40× high power lens. Each tumor was given a score according to the intensity of the staining (1-weak staining; 2-moderate staining; 3-strong staining) and the percentage of stained cells (0, staining value 0%–10%; 1, 11–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% positive cells). The total score was obtained by multiplying the proportion score and the stain score. The median was defined as cutoff values. Low LGALS3BP expression was defined as the score below the cutoff value, while high LGALS3BP expression was defined as the score above the cutoff value. Overall survival (OS) was defined as the periods from the initial surgery date to the date of death. Disease-free survival (DFS) was defined as the periods from the initial surgery date to the date of diseases recurrence or metastasis. Recurrence-free survival (RFS) was defined as the periods from the initial surgery date to the date of recurrence.

2.4. Cell culture and reagents

Human OSCC cell lines SCC4、Cal27、HSC3 and human normal keratinocyte cell HaCaT were kept in our lab and cultured in Dulbecco's modified Eagle's medium (DMEM) basal medium supplemented with 10% fetal bovine serum (FBS, Gibico) and 1% penicillin-streptomycin (Gibico-BRL Life Technologies; Gaithersburg, MD, USA). All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO_2. LY294002 was purchased from Selleckchem.

2.5. RNA extraction and realtime PCR (RT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) and reversed into cDNA using the PrimeScript™ RT reagent kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. Real-time PCR was performed on an ABI Vii7 dx Real-Time PCR System (Applied Biosystems Inc.) with SYBR PrimeScript RT-PCR kit (TaKaRa). Each sample was performed in triplicate. The relative amount of cDNA was calculated by relative CT methods using β-actin cDNA as controls. The sequences for qPCR primers used in this study were as follows:

LGALS3BP_Fw: AGGTACTTCTACTCCCGAAGGA
LGALS3BP_Rv: GGCCACTGCATAGGCATACA
β-actin_Fw: TTCCTGGGCATGGAGTCC
β-actin_Rv: CTCGTTACTAGAACTAGAAGT.

2.6. Cell transfection

All siRNA were purchased from GenePharma (Shanghai, China) and transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The target sequences against LGALS3BP were as follows:

siRNA-232_Fw: GCGUGAACGAUGGUGACAUTT,
siRNA-232_Rv: AUGUCACCAUCGUUCACGCTT;
siRNA-641_Fw: CCUGUCCAUCAGCGUGAAUTT,
siRNA-641_Rv: AUUCACGCUGAUGGACAGGTT;
siRNA-1425_Fw: GCCUUUGUGACAGACAGUUTT,
siRNA-1425_Rv: AACUGUCUGUCACAAAGGCTT.

For silencing LGALS3BP expression, short hairpin RNA against LGALS3BP was generated and cloned into the retroviral vector (pLVX-GFP). HEK293T cells were co-transfected with viral plasmids and packaging plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after transfection, culture medium was filtered through 0.45 μm filter and infected the cells of interest.

For over-expression, the full-length cDNA of LGALS3BP was amplified using primers:

LGALS3BP_Fw: tttgacctccatagaagattATGACCCCTCCGAGGCTCTT
LGALS3BP_Rv: gatccttgcggccgcggatcCTAGTCCACACCTGAGGAGT

Then, the purified PCR products were cloned into pCDH vector (pCDH-GFP) according to the manufacturer's instruction (Vazyme). LGALS3BP-pCDH vector or pCDH empty vector was transfected into HEK293T cells. After harvesting and concentrating, virus containing supernatants were added to cells of interest.

2.7. Western blotting

Western blotting was performed using the standard protocol. Cells

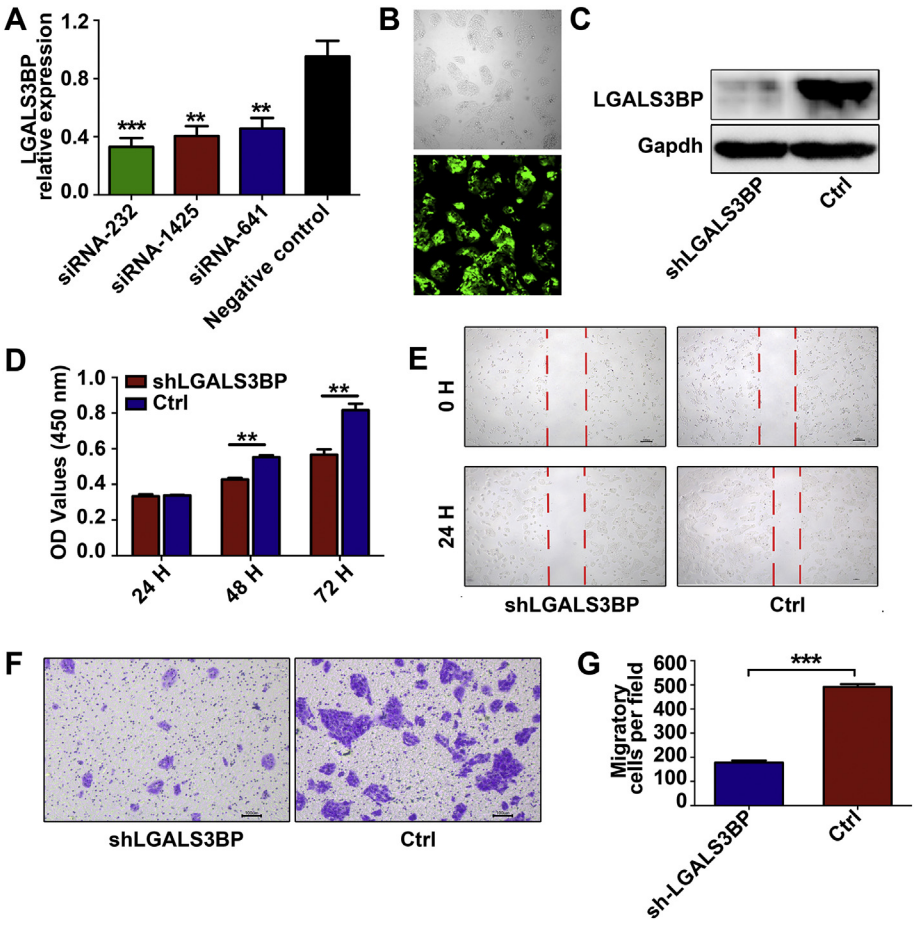

Fig. 3. Knockdown of LGALS3BP reduced cell proliferation and migration in SCC4 cells.
A) Relative LGALS3BP expression after transfection with control, siRNA-232, siRNA-1425, siRNA-641; B) Images of SCC4-shLGALS3BP cells under white light (upper) and fluorescence (below); C) Western blot analysis of LGALS3BP expression in SCC4-shLGALS3BP versus SCC4-Ctrl; D) CCK8 analysis of SCC4-shLGALS3BP versus SCC4-Ctrl; E) Wound healing analysis of SCC4-shLGALS3BP versus SCC4-Ctrl; F) Images of migrating SCC4-shLGALS3BP versus SCC4-Ctrl in trans-well assays; G) Quantification of migrating SCC4-shLGALS3BP versus SCC4-Ctrl in trans-well assays. Data was presented as mean ± S.D. ** $p < 0.01$, *** $p < 0.01$.

Table 3
Cox-regression analyses of overall survival (OS) for clinicopathological parameters in OSCC patients.

	Univariate analysis			Multivariate analysis		
	Hazard Ratio	95% CI	*p*	Hazard Ratio	95% CI	*p*
Age	4.642	1.870-11.520	**0.001**	4.325	1.488-12.567	**0.007**
Smoking	1.494	0.660-3.380	0.336	1.318	0.469-3.703	0.601
T stage	1.467	0.697-3.089	0.313	0.905	0.391-2.096	0.816
Differentiation	1.333	0.614-2.898	0.467	1.251	0.502-3.120	0.631
WPOI	2.180	0.915-4.856	0.080	1.061	0.385-2.927	0.909
Nerve invasion	1.158	0.441-3.042	0.765	0.999	0.363-2.748	0.998
Inflammation	0.710	0.336-1.500	0.369	0.804	0.326-1.983	0.636
LGALS3BP	9.613	3.282-28.160	< **0.001**	9.032	2.847-28.657	< **0.0001**

Table 4
Cox-regression analyses of disease-free survival (DFS) for clinicopathological parameters in OSCC patients.

	Univariate analysis			Multivariate analysis		
	Hazard Ratio	95% CI	*p*	Hazard Ratio	95% CI	*p*
Age	2.468	1.172-5.196	**0.017**	2.207	0.991-4.911	**0.053**
Smoking	1.283	0.578-2.849	0.540	1.960	0.782-4.914	0.151
T stage	1.045	0.514-2.217	0.903	0.589	0.277-1.251	0.169
Differentiation	2.129	0.960-4.722	0.063	1.936	0.807-4.642	0.139
WPOI	2.156	0.972-4.781	0.059	1.211	0.507-2.895	0.666
Nerve invasion	0.719	0.252-2.049	0.537	0.479	0.165-1.392	0.176
Inflammation	1.091	0.519-2.294	0.819	1.181	0.514-2.710	0.695
LGALS3BP	4.184	1.866-9.382	**0.001**	4.073	1.706-9.126	**0.002**

Table 5
Cox-regression analyses of recurrence-free survival (RFS) for clinicopathological parameters in OSCC patients.

	Univariate analysis			Multivariate analysis		
	Hazard Ratio	95% CI	*p*	Hazard Ratio	95% CI	*p*
Age	2.321	1.048-5.141	**0.038**	2.026	0.864-4.752	0.104
Smoking	0.851	0.323-2.241	0.744	1.274	0.438-3.707	0.657
T stage	1.194	0.559-2.553	0.647	0.765	0.345-1.697	0.510
Differentiation	2.597	1.053-6.408	0.038	2.372	0.881-6.392	0.088
WPOI	1.765	0.777-4.008	0.175	0.874	0.347-2.204	0.776
Nerve invasion	0.881	0.305-2.544	0.351	0.621	0.208-1.855	0.393
Inflammation	1.169	0.515-2.657	0.709	1.151	0.482-2.751	0.752
LGALS3BP	4.909	1.972-12.218	**0.001**	4.631	1.773-12.095	**0.002**

were lysed in ice-cold RIPA buffer containing proteinase/phosphatase inhibitors for 30 min. 40 μg protein from each sample was electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride membranes. Membranes were blocked with 3% bovine serum albumin and then incubated with primary and secondary antibodies. Antibodies against LGALS3BP were purchased from Proteintech. Antibodies against AKT, p-AKT, PI3K, p-PI3K, GAPDH, and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Protein expression was detected by ECL detection reagents.

2.8. Cell proliferation assay

Cells at a density of 3000 cells/well were plated in 96-well plates for 24 h, 48 h and 72 h. At indicated time intervals, cells were exposed to 10 μl of Cell Counting Kit-8 (Dojindo, Kyushu, Japan) for 2 h at 37 °C in 5% CO_2. The absorbance of optical density was measured at 450 nm using a Varioskan Flash Microplate Reader.

2.9. Wound healing assay

Cells were cultured in a 6-well plate and grown to 100% confluence. The cell-free wound was performed with a 10 μl sterile pipette tip and medium was changed to remove the debris. The images of cells migrating to the wound area were imaged at different time points using a phase-contrast microscope and captured by CAP Studio software.

2.10. Cell migration assay

Cell migration assay was carried out in a Transwell with 8 μm pore size chemotaxis chamber (Corning, New York, USA). 1×10^4 cells in 100 μl serum-free medium were cultured in the upper chambers. The medium with 10% FBS was employed as a chemoattractant in the lower chamber. Cells were cultured for 24 h, and cells migrated to the bottom side of the inserts were fixed with methyl alcohol and stained with crystal violet. The result was determined by counting the stained cells using optical microscopy (100× magnifications) in five randomly selected fields. Each experiment was carried out in triplicate wells and repeated at least three times.

2.11. Statistical analysis

The data were presented as the mean ± SD. Statistical analysis was performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). Correlation of LGAS3BP expression to the clinical parameters was evaluated by chi-square test. Statistical significance was determined by the two tailed Student's *t*-test. Kaplan-Meier survival was used for survival analysis. Prognosis was performed by univariable and multivariable Cox proportional hazard models. $p < 0.05$ were taken as indicative of statistically significant differences.

3. Results

3.1. LGALS3BP was over-expressed in OSCC

Our initial proteomic analysis of OSCC tissues and the adjacent non-tumor tissues using TripleTOF 4600 System showed that LGALS3BP, one of the central nods in protein-protein interaction network, was upregulated in tumor tissues (Fig. S1). In order to clarify the expression of LGALS3BP in OSCC tissues, we then analyzed public datasets Oncomine and found that LGALS3BP was highly expressed in cancers compared with normal tissues (Fig. 1A). Specifically, Oncomine contained 7 datasets comparing LGALS3BP expression between OSCC and their normal tissue parts, three of which displayed a significant LGALS3BP upregulation in cancers, with fold changes ranging from 2.215 to 2.890 (Fig. 1B). Although LGALS3BP expression at gene levels was confirmed by the datasets, the expression profile of LGALS3BP in OSCC at protein levels was unclear. Therefore, LGALS3BP expression levels in 75 human OSCC specimens and 64 adjacent normal mucosa tissues were explored by IHC. As shown in Fig. 1C and D, LGALS3BP was over-expressed in OSCC compared to normal mucosal epithelium, which was in accordance with public gene datasets.

As solid tumors are composed of heterogeneous cells, including cancer cells, immune cells, fibroblasts etc., the particular cell type that expresses LGALS3BP remains elusive. Therefore, LGALS3BP expression profiles between cancer cells, immune cells and cancer associated fibroblasts were analyzed. As shown in Fig. 1E, LGALS3BP was exclusively expressed in tumor cells, indicating that LGALS3BP had important roles in tumor cells. Furthermore, LGALS3BP expression in OSCC cell lines and human immortal keratinocyte cell line HaCaT cells was assessed. Both RT-PCR and western blot analysis showed that LGALS3BP was overexpressed in SCC4, Cal27 and HSC3 cells compared to HaCaT cells and SCC4 cells displayed the highest expression of LGALS3BP among the three OSCC cell lines (Fig. 1F, G). Taken together, LGALS3BP was overexpressed in cancer cells of OSCC.

3.2. LGALS3BP was an independent prognostic factor for OSCC

To investigate the clinical significance of LGALS3BP, we evaluated the LGALS3BP protein expression levels in 92 OSCC specimens by IHC. LGALS3BP was predominantly expressed in the cytoplasm of tumor cells (Fig. 2A, B, C, D). Table 1 displayed the distribution of the intensity and the percentage score of LGALS3BP IHC staining and the total scores were acquired by multiplying the intensity and the percentage scores. The patients were divided into two groups with a cutoff value of 8. As shown in Table 1, 45.65% (42/92) specimens exhibited high LGALS3BP expression, while 54.35% (50/92) specimens exhibited low LGALS3BP expression. Elevated expression levels of LGALS3BP were significantly associated with poorer clinicopathological features, including higher T stages ($p = .043$), more recurrence ($p = .002$) and less overall survival ($p < .0001$)(Table 2). Besides, our analysis of head and neck cancer patients in The Cancer Genome Atlas (TCGA) data also showed that LGALS3BP expression levels were upregulated in advanced

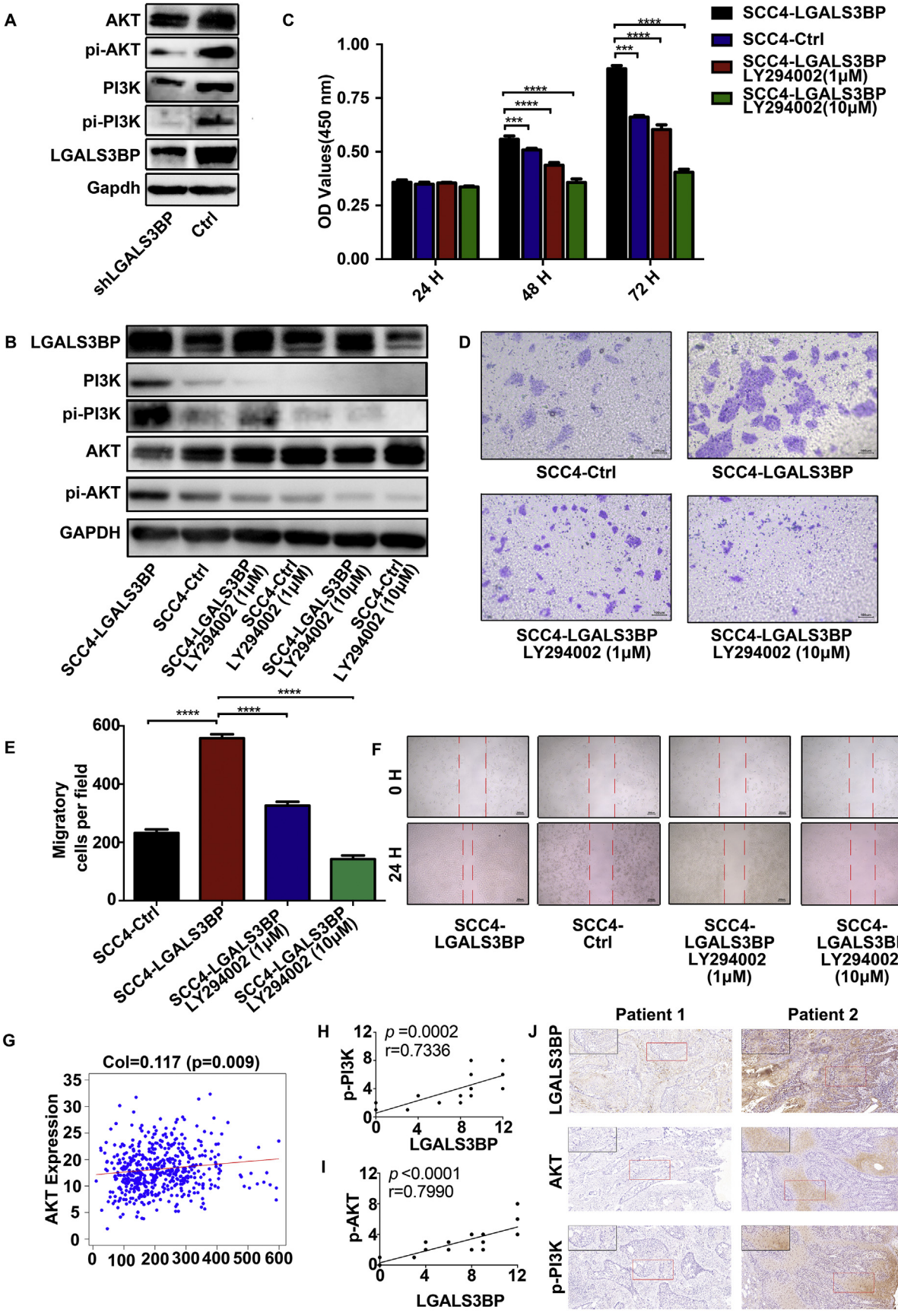

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Fig. 4. LGALS3BP regulated cell proliferation and migration by PI3K and AKT pathway in SCC4 cells.
A) Western blot analysis of phosphorylated PI3K and AKT expression in SCC4-shLGALS3BP versus SCC4-Ctrl; B) Western blot analysis of phosphorylated PI3K and AKT expression in SCC4-LGALS3BP and SCC4-Ctrl after treated with different doses of LY294002 for 24 h; C) CCK8 analysis of SCC4-LGALS3BP and SCC4-Ctrl after treated with different doses of LY294002 for 24 h; D) Images of migrating SCC4-LGALS3BP and SCC4-Ctrl cells after treated with different doses of LY294002 for 24 h in trans-well assays; E) Quantification of migrating SCC4-LGALS3BP and SCC4-Ctrl cells after treated with different doses of LY294002 for 24 h in trans-well assays; F) Wound healing analysis of SCC4-LGALS3BP cells and SCC4-Ctrl cells after treated with different doses of LY294002 for 24 h. G) The correlation of LGALS3BP and AKT m RNA levels in head and neck cancer patients in TCGA data base; H) Pearson analysis of correlation between LGALS3BP and p-PI3K expression levels using clinical samples; I) Pearson analysis of correlation between LGALS3BP and p-AKT expression levels using clinical samples; J) Representative images of IHC staining of LGALS3BP, p-AKT and p-PI3K in two patients. Data were presented as mean ± S. D; ***$p < 0.001$,**** $p < 0.0001$.

T grades (Fig. 2E). The results indicated that LGALS3BP was correlated with OSCC progression.

Then we evaluated the prognostic significance of LGALS3BP in OSCC. Kaplan-Meier survival curve analysis was performed to compare the clinical outcomes between LGALS3BP high expression group and low expression group. Compared to LGALS3BP low expression patients, patients with high expression had shorter OS, RFS and DFS ($p < 0.0001$, $p = .0002$, $p = .0002$)(Fig. 3F, G, H), indicating that high LGALS3BP expression predicted poorer clinical outcomes in OSCC patients.

Furthermore, in order to explore the prognostic values of LGALS3BP expression in OSCC patients, univariate and multivariate analyses were performed to determine the risks associated with OS, RFS and DFS. By the univariate analysis, we found that LGALS3BP expression and age were the statistically associated with patient's overall survival ($p < .001$, $p = .001$), recurrence free survival ($p = .001$, $p = .038$) and disease-free survival ($p = .001$, $p = .017$) (Table 3)(Table 4)(Table 5). In addition, multivariate Cox proportional regression analysis revealed that LGALS3BP expression was an independent prognostic indicator for OS ($p < .0001$), RFS ($p = .002$) and DFS ($p = .002$) of OSCC patients (Table 3)(Table 4)(Table 5).

3.3. LGALS3BP was responsible for OSCC proliferation and migration

Although LGALS3BP was shown to be a valuable prognostic factor in OSCC, its biological function in OSCC remains elusive. To explore the functional roles of LGALS3BP in OSCC, three candidate siRNA against LGALS3BP were screened to select the one that effectively knocked down LGALS3BP expression for further research (Fig. 3A). We then generated stable LGALS3BP-knockdown SCC4 cell lines using lentivirus-based shRNA targeting the selected LGALS3BP sequence. As shown in Fig. 3B, 90% SCC4 cells expressed GFP, indicating they were transfected with shRNA plasmids. Western blot analysis further confirmed that LGALS3BP was efficiently silenced (Fig. 3C).

Clinical data showed that elevated LGALS3BP expression levels were associated with higher T stages and LGALS3BP expression levels of head and neck cancer patients with advanced T stages were significantly higher than the other groups. Therefore, we first evaluated the proliferation rates of SCC4-ctrl and SCC4-shLGALS3BP by CCK8 and found that the growth rates of SCC4 were significantly reduced after silencing LGALS3BP (Fig. 3D). As tumors are notorious for their fatal propensity to metastasize, we then performed trans-well and wound healing assay to test the migration ability of SCC4-shLGALS3BP. As shown in Fig. 3F, the ability of cells migrating to lower channel was significantly decreased after silencing LGALS3BP. Compared to SCC4-ctrl, only 10% SCC4-shLGALS3BP cells were found in the lower channel. It was consistent with wound healing assay, which showed that the migration of SCC4 was markedly reduced by silencing LGALS3BP (Fig. 3E).

To test whether LGALS3BP has similar effects on other cell types, we generated stable LGALS3BP-knockdown CAL27 and HSC3 cells (Sup Fig. 2A). Consistently, silencing LGALS3BP impaired cell proliferation and migration in the two cell lines (Sup Fig. 2B, C, D). Collectively, the above results suggested that LGALS3BP promoted the proliferation and migration of OSCC cells.

3.4. LGALS3BP promoted OSCC proliferation and migration by PI3K/AKT pathway

Although the mechanism of the pro-tumor effects of LGALS3BP was unclear in OSCC, previous reports demonstrated that LGALS3BP transmitted signals via Akt, JNK and the Ras cascade to promote cell survival and proliferation in melanoma and liver cancers [22]. In addition, the PI3K/Akt signaling pathway was reported to be involved in LGALS3BP-mediated chemotherapy resistance in lung cancers [18]. Therefore, we first analyzed PI3K and AKT expression. As shown in Fig. 4A, LGALS3BP knockdown significantly decreased PI3K and AKT phosphorylation. To verify LGALS3BP regulated OSCC proliferation and migration by PI3K/AKT pathway, we treated SCC4-LGALS3BP, which constitutively expressed LGALS3BP with LY294002. LY294002 blocks PI3 kinase-dependent AKT phosphorylation and kinase activity. As shown in Fig. 4B, LGALS3BP overexpression increased the phosphorylation of PI3K and AKT, while LY294002 suppressed PI3k and AKT phosphorylation in a dose-dependent manner.

In order to clarify whether LGALS3BP promoted cell proliferation and migration via PI3K/AKT pathway, we treated SCC4-LGALS3BP with LY294002. CCK8 analysis showed that elevated LGALS3BP expression promoted cell proliferation, which was abrogated by LY294002 in a dose dependent manner (Fig. 4C). In addition, the rates of SCC4-LGALS3BP migration were remarkedly enhanced compared to SCC4-Ctrl. However, the rates were reduced when SCC4-LGALS3BP cells were dose dependently treated with LY294002 (Fig. 4D). The effects of LGALS3BP on migration were further proved by wound healing assay (Fig. 4F). Finally, we evaluated whether LGALS3BP regulated PI3K/AKT pathway in clinical samples. As shown Fig. 4H, I and J, there were strong correlations between LGALS3BP and pi-PI3K and pi-AKT expression levels in OSCC patients ($n = 20$, $r = 0.7336$, $p = .0002$; $n = 20$, $r = 0.7990$, $p < .0001$). Similarly, analysis of TCGA database revealed that the expression of LGALS3BP was minorly positively correlated with that of AKT ($r = 0.117$, $p = .009$). The results showed that LGALS3BP regulated OSCC proliferation and migration by PI3K/AKT pathway.

4. Discussion

The aim of our study was to identify a candidate molecular marker associated with poor outcomes of OSCC and its regulatory pathways. OSCC is still a challenge in the field of head and neck cancers and lack of valuable biomarkers that predict aggressiveness and recurrence risk made this scenario even more dismal. In this study, we found that LGALS3BP was overexpressed in OSCC. Moreover, the expression levels of LGALS3BP were associated with poor clinical characteristics and an independent prognostic indicator for OS, RFS and DFS in OSCC. We also provided evidence that LGALS3BP regulated OSCC proliferation and migration via PI3K/AKT pathway. Therefore, our findings of LGALS3BP are important for developing personalized treatments and effective cancer therapies.

Serum tumor-associated antigens (TAAs) have been extensively studied for early cancer detection [23,24]. As they can be easily detected because of high concentration and long-time stability, LGALS3BP was initially discovered as a tumor-secreted antigen in human breast cancer [25]. And it is overexpressed in many types of cancer tissues,

including colorectal cancer [20], hepatocellular carcinoma [26], pleural mesothelioma [15], pancreatic carcinoma [16], non-small cell lung carcinoma [8] and neuroblastoma [19]etc. Yu et al. recently showed that concentration of LGALS3BP was elevated in the saliva of OSCC patients [27]. However, they failed to study its prognostic values and cellular functions in OSCC. In our study, we found that LGALS3BP was solely expressed on tumor cells. In addition, both IHC and Oncomine analysis (including Toruner Head-Neck, Estilo Head-Neck and Pyeon Multi-cancer dataset) showed that LGALS3BP was overexpressed in tumors than the matched adjacent non-tumor tissues, indicating it might play an important role in tumor progression.

The effects of LGALS3BP on clinical prognosis was still controversial. Some reports showed that LGALS3BP overexpression was associated with poor clinical outcomes, while some reports displayed that LGALS3BP was a favorable factor on cancer prognosis [9,11,22,28]. However, no information is available regarding the association between LGALS3BP and clinical outcomes in OSCC. Our results displayed that high expression of LGALS3BP was associated with advanced T stages and more recurrence. The patients with high level expression of LGALS3BP had shorter OS, DFS and RFS. Multivariate analysis showed that LGALS3BP was an independent prognostic marker for OS, DFS and RFS. The aforementioned results indicated that LGALS3BP was important for OSCC progression.

By phenotypic and functional analysis, we further found that LGALS3BP regulated OSCC proliferation and migration. Knocking down LGALS3BP permanently in OSCC cells led to a reduction of cell proliferation and migration, which were consistent with Endo H and Fukamachi M, et al.'s findings [29,30]. However, LGALS3BP was found not to be significantly associated with metastasis in our clinical study. The reason of the inconsistent may lie in the limited number of clinical samples enrolled in our research. Therefore, more patients should be gathered in the further research.

LGALS3BP, a secretory glycoprotein, binds to important molecules associated with adhesion, such as galectin-3, galectin-1, collagen and fibronectin and reported to be regulated by multiple coagulation factor deficiency protein 2 and miR-596 in OSCC [29–33]. Upon binding specific ligands on cell membranes, signals are transmitted via AKT, JNK and the Ras cascades, which affect cell proliferation, migration, apoptosis and chemotherapy sensitive [34–36]. In addition, we found that LGALS3BP expression levels affected PI3K and AKT phosphorylation. PI3K/AKT was a classical signaling pathway and its activation led cell growth, EMT and tumor metastasis [37–39]. PI3K/AKT has been shown to be highly activated in OSCC due to genetic and epigenetic alterations, which means this pathway plays a central role in cell growth, apoptosis, survival and differentiation in OSCC [37,40]. In this study, we found that the proliferation and migration rates were significantly reduced even when LGALS3BP was overexpressed after the cells were treated with PI3K inhibitor LY294002. As the promoting effects of LGALS3BP on cellular functions were abolished by LY294002 treatment, we concluded LGALS3BP regulated cell proliferation and migration via PI3K/AKT pathway. Furthermore, clinical samples showed that the expression of LGALS3BP was positively correlated with pi-PI3K and pi-AKT expression. As the expression levels of pi-AKT were not available in TCGA datasets, we only compared the total mRNA levels between LGALS3BP and AKT using TCGA data base. However, the correlation coefficient between LGALS3BP and AKT was minor, indicating that hyper-activation of the PI3K/AKT pathway in OSCC was crucial for LGALS3BP regulated oral cancer development.

5. Conclusion

In this study, we showed that LGALS3BP was overexpressed in OSCC. Overexpression of LGALS3BP was associated with unfavorable clinical characteristics and could be served as an independent prognostic marker for OS, RFS and DFS in OSCC. Moreover, LGALS3BP promoted OSCC cell proliferation and migration by activation the PI3K/AKT axis. Thus, LGALS3BP may serve as a novel biomarker for OSCC diagnosis, and targeting LGALS3BP exhibited potential application in OSCC treatments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109359.

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