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Sonic Hedgehog Signalling Activation Contributes to ALCAM Over-Expression and Poor Clinical Outcome in Patients with Oral Squamous Cell Carcinoma

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Objective: *To investigate the Sonic Hedgehog (SHH) signalling molecules and activated leukocyte cell adhesion molecule (ALCAM) expression in the mechanisms regulating invasion and metastasis in oral squamous cell carcinoma (OSCC).*

Methods: *The expressions of SHH signalling molecules Gli family zinc finger 1/2 (Gli1/Gli2), as well as ALCAM expression, was analysed in 101 OSCC patients by immunohistochemistry. The potential relationship between Gli1/Gli2 and ALCAM in regard to invasion and metastasis were studied by western blot, invasion and wound-healing assays.*

Results: *Gli1, Gli2 and ALCAM were expressed in 54.5%, 49.5% and 47.5% of the 101 OSCC specimens, respectively. High expression of ALCAM was associated with shorter survival in the patient population (P = 0.018), which was independent of other clinical parameters. Notably, when both ALCAM expression and positive nodal status were considered, an enhanced prediction of clinical outcomes was achieved (P = 0.001). In OSCC cell lines, down-regulation of ALCAM resulted in reduced cell invasion and metastasis. Importantly, SHH activation increased the half-life of ALCAM leading to ALCAM accumulation and increased cell invasion and migration.*

Conclusion: *ALCAM over-expression in OSCC is an independent prognostic factor for OSCC patients. Its over-expression may be the result of the activation of the SHH signalling pathway and contributes to OSCC progression.*

Key words: *ALCAM, Gli1, Gli2, invasion, migration, OSCC*

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Distant metastases and local recurrences are the major causes of cancer-related death. Most patients with localised lymph node metastases die within 5 years. To improve treatment outcomes for OSCC, novel biomarkers and potential mechanisms that regulate invasion and metastasis of OSCC cells must be identified[1].

ALCAM is a transmembrane glycoprotein and a member of the Ig superfamily. It is involved in important pathophysiological processes, such as mediating cell-to-cell adhesion, invasion and metastasis of tumour cells and maintaining the stemness of cancer stem cells (CSCs)[2]. Its expression is variable in malignancies. In some cancers, it is over-expressed, such as melanoma, esophageal, gynaecologic, prostate, and pancreatic cancers. However, in prostate, breast and ovarian cancer,

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the expression levels are low[3-6]. In OSCC, the expression level of ALCAM is also contradictory[7]. In light of the contradicting reports regarding ALCAM expression in OSCC, one of the aims of this study was to analyse membranous ALCAM expression in OSCC.

The SHH signalling pathway plays a fundamental role in tumour initiation and/or progression to more advanced tumour stages, embryonic development, maintenance of CSCs and metastasis[8]. Its abnormal activation is closely associated with tumorigenesis, migration, metastasis, cancer stemness and chemoresistance[9,10]. Gli1 and Gli2 act as the primary transcriptional activators. When the SHH pathway is activated, nuclear localisation of Gli transcription factors drives expression of SHH target genes to control cell proliferation, differentiation, invasion and migration[11].

However, the exact role of the SHH pathway in OSCC is still unclear. For example, Wang et al showed a close association between nuclear Gli1 over-expression and cancer recurrence and lymphatic metastasis, indicating a poor prognosis in 40 patients with OSCC[12]. However, Yan et al reported that expression of Gli2, but not Gli1, is strongly associated with a poor clinical outcome of OSCC patients[13]. Most of the studies focused on Gli1, and the number of samples included in these studies was relatively small. In addition, the correlation of SHH pathway activation and the expression level of ALCAM protein has not been reported.

In the present study, we found that membranous ALCAM could serve as a promising biomarker for OSCCs, which is better than Gli1 and Gli2. The SHH signalling pathway upregulated the accumulation of ALCAM protein by extending its half-life, which caused comparatively stronger invasive and migratory activities.

Materials and methods

Cell lines and cell culture

The OSCC cell lines included: HN4, HN6, HN12, HN13, HN30 and CAL27. Normal epithelial cells of gingival tissues were obtained from patients with impacted teeth extraction.

The OSCC cell lines were maintained in Dulbecco's Minimum Essential Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Normal epithelial cells were cultured in keratinocyte serum-free medium (KSF; GIBCO-BRL) with 0.2 ng/ml recombinant epidermal growth factor (rEGF; Invitrogen). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Patients and specimens

The tissue microarray samples were obtained from the paraffin-embedded tissue of 101 patients with OSCC who had undergone surgical treatment when first diagnosed with OSCC between 1989 and 1993. The patients had primary OSCC and no distant metastases. The patients received no prior treatments. The detailed information of the 101 patients was obtained to analyse the correlation between the expression levels of Gli1/Gli2 and ALCAM and clinical parameters. Samples were stained with hematoxylin and eosin (H&E) to analyse the pathological differentiation and pathological diagnosis, which were confirmed by two experienced pathologists. Pathological differentiation and clinical stage were respectively determined according to the World Health Organization Classification of Tumours and the TNM classification system of the International Union Against Cancer (1988). The patients signed a written informed consent, and the study was approved by the Medical Ethics Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

Immunohistochemistry

The tissues were fixed with formalin, embedded in paraffin, then cut into 4 µm sections. The samples were incubated with rabbit polyclonal ALCAM antibody (1:150; Epitomics - an Abcam Company, Burlingame, CA, USA), rabbit polyclonal Gli1 antibody (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit polyclonal Gli2 antibody (1:2000; Epitomics) at 4°C overnight, followed by incubation with a biotinylated secondary antibody and staining with Diaminobenzidine (DAB; Dako, Denmark). The staining index was quantitatively divided into scores of 0, 1, 2, and 3, according to the intensity of staining. In this study, the scores representing the percentage of positive epithelial cells were obtained and expressed as 0 to 100% and were analysed by two experienced pathologists.

The total scores were obtained by multiplying the staining index by the percentage of positive epithelial cells. The expression levels of Gli1, Gli2 and ALCAM were divided into high and low levels based on a median score of 2.55, which was the cut-off value for determining Gli1, Gli2 and ALCAM low or high expression. Thus, a value < 2.55 was considered low expression, and a value ≥ 2.55 was considered high expression.

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Real-time quantitative PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers are as follows: Gli1 sense, 5′- ACT ACC TGC TTC GGG CAA G-3′ and anti-sense, 5′- GAT ACT CGG CTC GGC TTC TC-3′; Gli2 sense, 5′- ACC GCT GCT CAA AGA GAA TG-3′ and antisense, 5′- GTC TTG ATG GCT CTG ACG TG-3′; ALCAM sense, 5′- CCT TGC CGA CTT GAC GTA CC-3′ and antisense, 5′- TGC CTC AAA CAC GTT GTC CT-3′; β-actin sense, 5′- GAT GAG ATT GGC ATG GCT TT-3′ and antisense, 5′-GAG AAG TGG GGT GGC TT-3′. Data were analysed using the 2-ΔΔCT method and normalised by β-actin expression in each sample.

Transfection of pLV-shALCAM plasmids

For silencing ALCAM expression, two oligo DNAs (sh874-5'-AAGCCCGATGGCTCCCCAGTA-3', Sh1747-5'-CCCGTGTCATGCACAATAT-3), which generated the small hairpin RNA (shRNA) targeting to ALCAM and one control scramble shRNA constructs (scrambled-5'-TTCTCCGAACGTGTCACGT-3) for the ALCAM cDNA sequence (Gene bank NM_001627.3) were synthesised and cloned into lentivirus vectors (pLVTHM) to form constructed pLVTHM-shRNA plasmids. To establish stably ALCAM-silent HN12 and HN13 cells, the cells were seeded into 6-well plates 24 h prior to the transfection, and then incubated with pLVTHM (scramble), pLVTHM containing the sequence 1 (pLV-sh874) or pLVTHM containing the sequence 2 (pLV-sh1747) in serum-free transfection medium (GIBCO) for 24 h, and replaced with fresh complete DMEM and cultured additionally for 48 h.

Plasmid construction and transfection

To construct the ALCAM expression vector, the open reading frame (ORF) of human ALCAM cDNA was cloned into the eukaryotic expression vector pcDNA3.0 (Invitrogen). The forward primer used for the amplification of the ORF of the cDNA was 5'- TGT AAG CTT TGT TCT GGG AGT TGCT -3, containing a HindIII site; the reverse primer was 5'-TAT GAA TTC TGT ACG GGA GGC CAC CAG CGC - 3, containing an EcoRI site without the stop codon. The amplified product of the ALCAM gene was purified, digested and ligated into the respective HindIII and EcoRI sites in the pcDNA3.0 vector. Gli2 plasmid was purchased from Addgene (pCS2-MT Gli2ΔN; pCS2-MT Gli2 FL). Cells at 60% to 80% confluence were transfected with various plasmids using lipofectamineTM 2000 (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions.

Western blot analysis

Cells were harvested in SDS lysis buffer (Beyotime, Haimen, China), and cell lysates were electrophoresed through 8% to 10% polyacrylamide gels and transferred to a PVDF membrane. The membranes were incubated with the desired primary antibody for Gli1 (1:1,000; Cell Signaling Technology and 1:200; Santa Cruz Biotechnology),Gli2 (1:1,000), ALCAM(1:1,000) (both from Abcam) and β-actin (1:5,000; Sigma-Aldrich) overnight at 4°C, followed by incubation with the appropriate secondary antibody for 1 h at room temperature. The immune-reactive bands were visualised by the Odyssey Infrared Imaging System (BD Biosciences, San Diego, CA, USA). Detection of β-actin was used as a loading control.

Invasion and wound-healing assays.

For the *in vitro* invasion assays, a total of 8×10^4 cells in 200 μl serum-free DMEM medium were plated onto BD BioCoat Matrigel Invasion Chambers (8 μm pore size; BD Biosciences), and the lower chamber was immediately filled with 500 μl of DMEM with 10% FBS as a chemo-attractant. After 24 h of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, the membranes were then fixed with methanol and stained with 0.2% crystal violet. For the wound-healing experiments, the cells were plated in six well plates, transfected as indicated, and cultured to confluency. Cells were serum-starved and scraped with a P200 tip (time 0), and images (five fields) were captured after 48 h.

Statistical analyses

In this study, the results of the real-time PCR were compared using the Student *t* test. One-way analysis of variance (ANOVA) was used to compare three groups. Kaplan-Meier analysis was used to estimate the Gli1/Gli2 and ALCAM expression. The Cox proportional hazards model was used for univariate and multivariate analyses of the prognosis of patients. The spearman's rank correlation coefficient test was used to examine the correlations among the expression levels of Gli1/Gli2 and ALCAM. In this study, all computation was accomplished using SPSS version 11.0 software and GraphPad Prism, and all statistical tests were two-sided. $P < 0.05$ was considered statistically significant.

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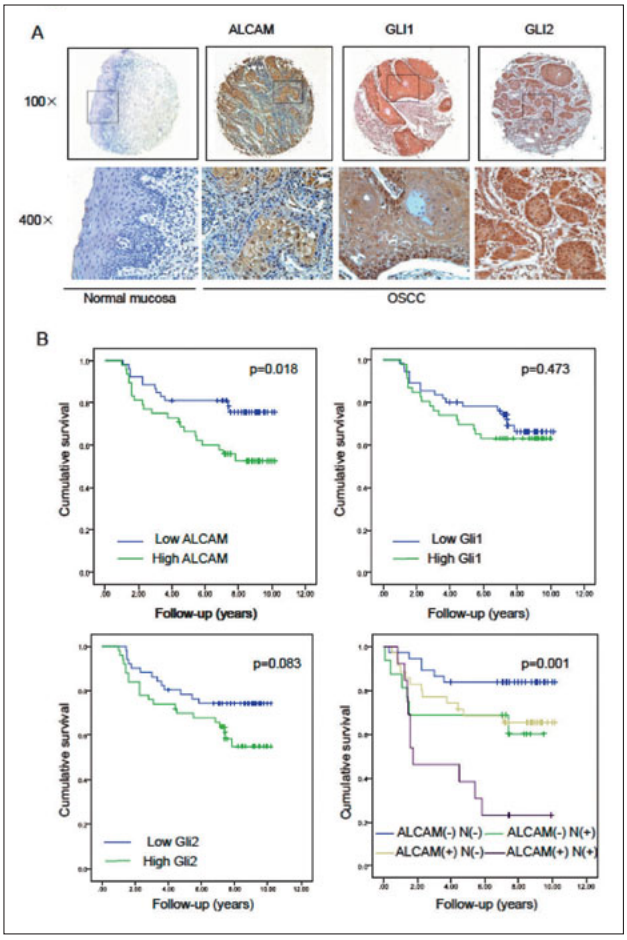

Fig 1 Compared with Gli1and Gli2, ALCAM was a better predictor of OSCC patients' poor prognosis. ALCAM expression, combined with nodal status, provides a better stratification for patients with OSCC. **A)** ALCAM, Gli1 and Gli2 proteins were barely expressed in normal oral mucosa. Gli1 and Gli2 was mainly expressed in nucleus of the cancer cells. ALCAM was detected in the cytoplasm and cytomembrane of almost all the cancer cells; **B)** The Kaplan–Meier survival curve prepared using the ALCAM scores showed that the ALCAM expression level was associated with a patient's prognosis ($P = 0.018$). There was no association between the level of expression of Gli1 and patient prognosis (Kaplan–Meier, $P = 0.473$). A marginal association between the level of expression of Gli2 and patient prognosis was noted (Kaplan–Meier, $P = 0.083$). The log-rank test prepared using both ALCAM scores and lymph node metastasis status (N stage) showed that this model might provide a better stratification for patients with OSCC ($P = 0.001$).

Results

ALCAM expression, rather than Gli1 and Gli2, is strongly associated with a poor clinic outcome of OSCC patients

Firstly, we examined the expression levels of Gli1/Gli2 and ALCAM in OSCC clinical samples, and then analysed the correlation between of Gli1/Gli2 and ALCAM with clinic-pathological features. A tissue microarray containing 101 OSCC specimens was assessed for the expression levels of Gli1/Gli2 and ALCAM by means of immunohistochemistry. ALCAM was not detected in the basal layer of the normal oral mucosa. ALCAM was localised mainly on the membrane of the cancer cells, and occasionally in the cytoplasm. Gli1 and Gli2 as main transcription factors were barely expressed in the basal layer of the normal oral mucosa. However, they were highly expressed in the nucleus, occasionally in the cytoplasm, in the cancer cells (Fig 1A).

A total of 46 (45.5%) of the 101 OSCC samples showed no Gli1 expression and 55 (54.5%) showed some level of Gli1 expression including 47 (46.5%) with moderate expression and eight (8%) with strong expression. A total of 51 (50.5%) of the 101 OSCC samples showed no Gli2 expression, while 50 (49.5%) showed Gli2 expression, including 40 (39.6%) with moderate expression and 10 (9.9%) with strong expression. Fifty-three (52.5%) of the 101 OSCC samples showed no ALCAM expression and 48 (47.5%) showed ALCAM expression, including 34 (33.7%) with moderate expression and 14 (13.8%) with strong expression. Gli1 expression was associated with tumour differentiation status ($P = 0.038$) and lymph node metastasis ($P = 0.01$). Gli2 expression was more frequently observed in tumours with moderate and poor differentiation ($P = 0.02$), advanced tumour stages ($P = 0.04$), and cervical lymph node metastasis ($P = 0.009$). ALCAM expression was only associated with lymph node metastasis ($P = 0.018$) (Table 1).

Patients whose tumours expressed ALCAM showed a significantly poorer overall survival rate than patients whose tumours expressed no ALCAM (Fig 1B, $P = 0.018$ by log-rank test). Five years after surgery, 81.1% (95% CI: 70.5 – 91.7) of the patients whose tumours expressed no ALCAM survived compared with only 66.5% (95% CI: 53.2–79.8) of those whose tumours expressed ALCAM ($P < 0.05$). In contrast, the survival rate was similar between the patients whose tumours expressed Gli1/Gli2 and those with tumours that lacked Gli1/Gli2 expression (Fig 1B, $P = 0.473$ for Gli1 and $P = 0.083$ for Gli2, respectively, by log-rank test).

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Table 1 Demographic characteristics of the patient population by Gli1, Gli2 and ALCAM expression.

Characteristic	N (%)	GLI1 expression		P	GLI2 expression		P	ALCAM expression		P
		+			-	+		-	+	
		No. %	No. %		No. %	No. %		No. %	No. %	
All patients	101	46 45.5	55 54.5		51 50.5	50 49.5		53 52.5	48 47.5	
Age				0.244			0.260			0.698
< 60y	59 (58.4%)	24 40.7	35 59.3		27 45.8	32 54.2		30 50.	29 49.2	
≥ 60y	42 (41.6%)	22 52.4	20 47.6		24 57.1	18 42.9		23 54.8	19 45.2	
Gender				0.098			0.925			0.262
Male	53 (52.5%)	20 37.7	33 62.3		27 50.9	26 49.1		25 47.2	28 52.8	
Female	48 (47.5%)	26 54.2	22 45.8		24 50.0	24 50.0		28 58.3	20 41.7	
Smoking history				0.590			0.248			0.214
No	63 (62.4%)	30 47.6	33 52.4		29 46.0	34 54.0		39 61.9	24 38.1	
Yes	38 (37.6%)	16 42.1	22 57.9		22 57.9	16 42.1		18 47.4	20 52.6	
Alcohol history				0.726			0.109			0.730
No	72 (71.3%)	32 44.4	40 55.6		40 55.6	32 44.4		37 51.4	35 48.6	
Yes	29 (28.7%)	14 48.3	15 51.7		11 37.9	18 62.1		16 55.2	13 44.8	
TNM stage				0.625			0.040			0.990
I/II	49 (48.5%)	20 40.8	29 59.2		30 61.2	19 38.8		26 53.1	23 46.9	
III	33 (32.7%)	17 51.5	16 48.5		13 39.4	20 60.6		17 51.5	16 48.5	
IV	19 (18.8%)	9 47.4	10 52.6		6 31.6	13 68.4		10 52.6	9 47.4	
Disease site				0.462			0.568			0.551
Tongue	78 (77.2%)	35 44.9	43 55.1		41 52.6	37 47.4		42 53.8	36 46.2	
Cheek	12 (11.9%)	4 33.3	8 66.7		5 41.7	7 58.3		5 41.7	7 58.3	
Gingiva	10 (9.9%)	6 60.0	4 40.0		4 40.0	6 60.0		6 60.0	4 40.0	
Base of tongue	1 (0.5%)	0 0	1 100.0		1 100.0	0 0		0 0	1 100.0	
Others (lip, floor of mouth)	1 (0.5%)	1 100.0	0 0		0 0	1 100.0		0 0	1 100.0	
Lymph node metastasis				0.01			0.009			0.018
pN0	73 (72.3%)	39 53.4	34 46.6		31 42.5	42 57.5		33 45.2	40 54.8	
pN1-pN2	28 (27.7%)	7 25.0	21 75.0		20 71.4	8 28.6		20 71.4	8 28.6	
Pathological differentiation				0.038			0.020			0.175
I	64 (63.4%)	36 56.3	28 43.8		39 60.9	25 39.1		38 59.4	26 40.6	
II	26 (25.7%)	7 26.9	19 73.1		9 34.6	17 65.4		10 38.5	16 61.5	
III	11 (10.9%)	6 54.5	5 45.5		3 27.3	8 72.7		5 45.5	6 54.5	

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Table 2 Cox proportional hazards regression models in estimating the overall survival

Characteristics	*P*	Hazard Ratio	95% CI
Age (< 60, > 60)	0.096	0.096	0.255 – 1.118
Sex (male, female)	0.116	0.569	0.282 – 1.150
Smoking history	0.067	1.480	0.972 – 2.252
Alcohol history	0.226	1.289	0.855 – 1.945
Pathology grade	0.006	1.894	1.204 – 2.978
TNM stage	0.010	1.731	1.138 – 2.634
N stage	0.003	2.749	1.400 – 5.398
GLi1	0.475	1.277	0.652 – 2.503
GLi2	0.013	1.844	1.140 – 2.983
ALCAM	0.022	2.283	1.129 – 4.615
Multivariate analysis			
TNM stage	0.133	1.441	0.895 – 2.319
N stage	0.019	2.364	1.152 – 4.851
Pathology grade	0.033	1.749	1.045 – 2.928
ALCAM	0.048	2.095	1.006 – 4.365
GLi2	0.516	1.190	0.704 – 2.011
Multivariate analysis			
N stage	0.001	3.005	1.525 – 5.920
Pathology grade	0.014	1.869	1.134 – 3.081
ALCAM	0.033	2.175	1.064 – 4.445

CI, confidence interval; N, lymph node; TNM, tumour-lymph node-metastasis classification.

In the univariate analysis, we found that the pathology grade, TNM stage, N stage, ALCAM and Gli2 expression were associated with patient overall survival. In the multivariate analysis, we found only N stage ($P = 0.001$), pathology grade ($P = 0.014$) and ALCAM ($P = 0.01$) were independent risk factors for overall survival in the patient population (Table 2). Considering nodal stages established clinical predictors for poor outcome in OSCC patients, we combined nodal status and ALCAM expression in one prognostic model and found this model might provide a better stratification for patients with OSCC (Fig 1B, $P < 0.0001$, log-rank test). A total of 61.5% of the patients whose tumours showed ALCAM expression and lymph node metastasis died 5 years after surgery, compared with 16.2% of those whose tumours expressed no ALCAM and had no lymph node metastasis ($P = 0.001$).

Expression levels of ALCAM and Gli1/Gli2 are correlated not only in OSCC, but also in cell lines

Studies have shown that the expression of ALCAM and Gli1/ Gli2 plays an important role in tumorigenesis[14-16]. For this reason, we ascertained whether the expression level of ALCAM was correlated with activity of the SHH pathway in OSCC. Correlations between Gli1/Gli2 and ALCAM expression patterns were analysed using Spearman's correlation test, and the results are present-

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Table 3 Association between expression of Gli1, Gli2 and ALCAM in patients with OSCC by Spearman's correlation analysis.

Molecule	Gli1		Gli2	
	Association coefficient (R)	*P* value	Association coefficient (R)	*P* value
ALCAM	0.235*	0.018	0.289**	0.003

R, Spearman's correlation coefficient; *, Statistically significant values ($P < 0.05$); **, Statistically significant values ($P < 0.01$).

ed in Table 3. These results showed that the ALCAM protein expression level was positively correlated with Gli1(r = 0.235, $P = 0.018$) and Gli2(r = 0.289, $P = 0.003$). We also determined the expression levels of ALCAM and Gli1/Gli2 in the OSCC cell lines and primary normal epithelial cells. Six OSCC cell lines and normal epithelial cell were chosen. Western blot analysis showed that the Gli1/Gli2 protein was over-expressed in the HN12 cells. ALCAM was high expressed in all OSCC cell lines (Fig 2A). The mRNA level of Gli1/Gli2 in the HN12 cells was higher than that noted in the other OSCC cell lines and normal epithelial cells. The mRNA level of ALCAM exhibited no obvious change in all cell lines (Figs 2B to D). The cell lines also showed correlation between these proteins. In conclusion, ALCAM and Gli1/Gli2 displayed a certain correlation in the cell lines and tissues.

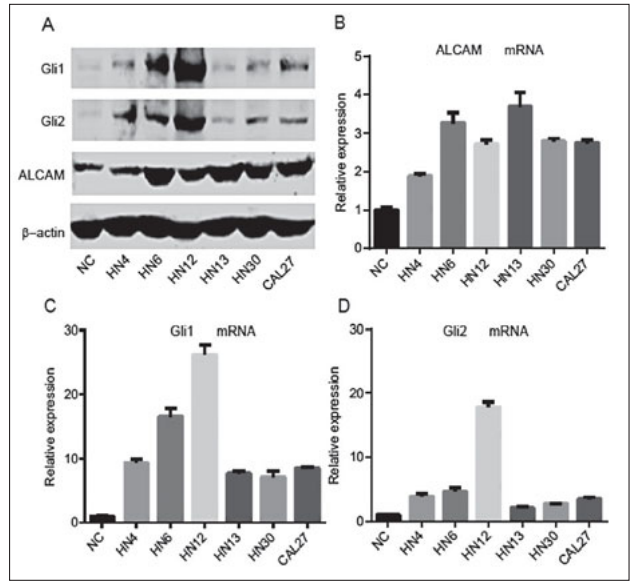

Fig 2 The mRNA and protein expression of ALCAM and Gli1/Gli2 were detected in cell lines. **A)** ALCAM, Gli1 and Gli2 protein levels were higher in OSCC cells than in normal epithelial cell. Compared with the ALCAM protein level, the variation in Gli1 and Gli2 protein levels was large among the different OSCC cells; **B to D)** ALCAM, Gli1 and Gli2 mRNA levels were higher in OSCC cells than that noted in normal epithelial cell, and the differences were significant ($P < 0.05$).

The SHH signalling pathway regulates the expression of ALCAM protein and mediates invasion and migration activities of the OSCC cells

The plasmids of Gli1 and Gli2 were transfected into the HN13 cells. Gli1/Gli2 increased the expression level of ALCAM protein (Fig 3A). However, the mRNA level of ALCAM did not have a statistically significant difference between the Gli1 or Gli2 plasmid transfection group and the control group (Fig 3C).

SHH recombinant protein was also used to activate this pathway. The expression level of ALCAM protein increased gradually over time. The level of ALCAM protein at 24 h was almost twice as much as that of control group (Fig 3B). However, the expression level of ALCAM mRNA at 12 or 24 h did not change (Fig 3D). The wound healing (Fig 3E) and Transwell assays (Fig 3F) showed that the invasion and migration activities of the HN13 cells increased. These abilities in the HN12 cells also showed similar results.

GANT61 can directly inhibit the DNA binding capacity of Gli and inhibit Gli-mediated gene transactivation[17,18]. GANT61 was used to inhibit the pathway. As shown in Figure 4A, the expression level of ALCAM protein obviously decreased with increased concentration of GANT61. The mRNA level of ALCAM was not obviously changed. The wound healing and Transwell assays showed that the invasion and migration activities of the HN13 cells were reduced. HN12 cells showed a similar situation. The results showed that the SHH signalling pathway could enhance invasion and migration activities of the OSCC cells by up-regulating the expression of ALCAM protein.

ALCAM does not regulate the activity of the SHH signalling pathway

To analyse whether ALCAM regulates the activity of the SHH signalling pathway, we knocked down ALCAM expression in the HN12 and HN13 cells, and found that

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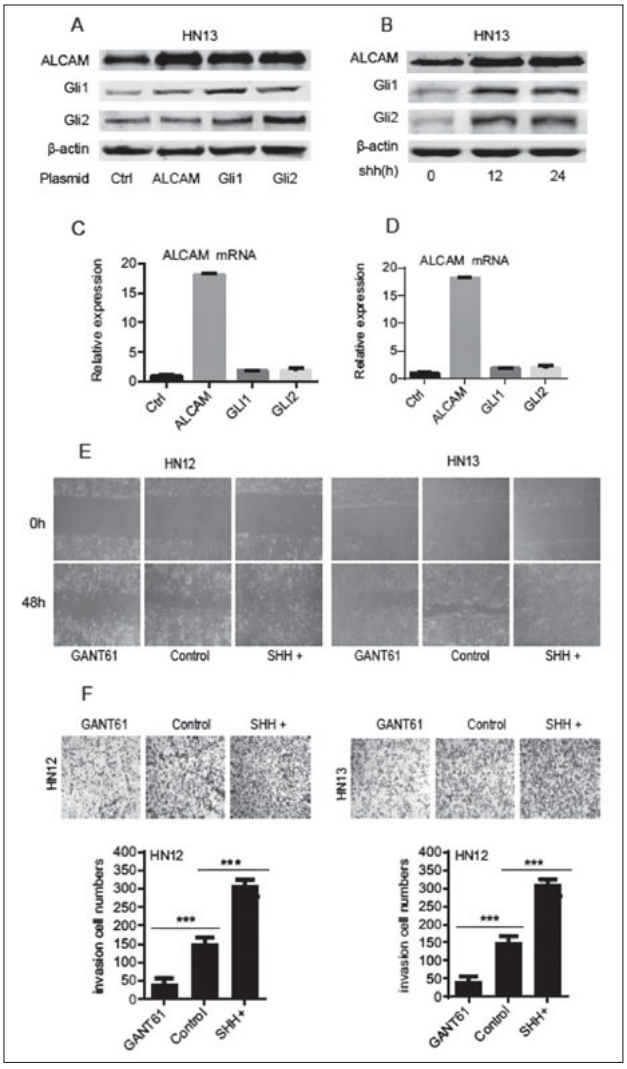

Fig 3 SHH signalling pathway activation enhances invasion and migration activities of the OSCC cells. **A)** After transfecting of the Gli1 and Gli2 plasmids in the HN13 cells, the ALCAM protein level was increased; **B)**. SHH recombinant protein could increase the ALCAM protein level in a time-dependent in the OSCC cells; **C)**. The mRNA level of ALCAM did not have a statistically significant difference between the Gli1 or Gli2 plasmid transfection group and the control group; **D)** There was no statistically significant difference in the mRNA level of ALCAM after SHH recombinant protein stimulation; **E)** Wound-healing assays showed that SHH recombinant protein stimulated cell migration; **F)** Representative image of cells that migrated through the filter, stained with crystal violet, and imaged at the same magnification (upper images). Absolute quantifications of the cells that invaded through the Transwell (lower graphs).*** means, $P < 0.01$

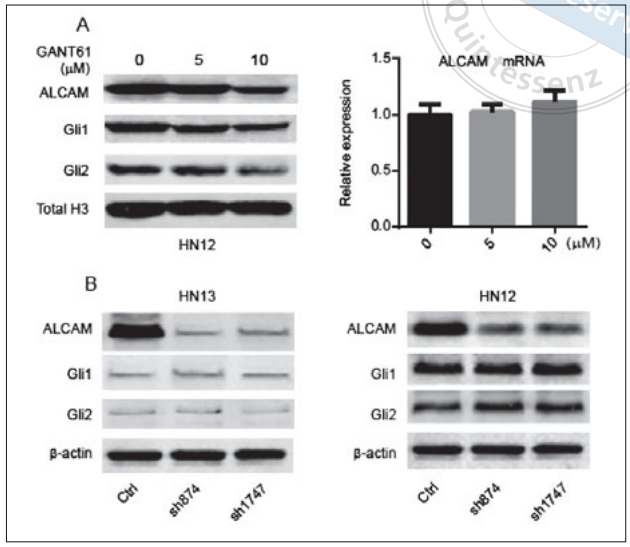

Fig 4 Inhibition of the SHH signalling pathway reduces the expression level of ALCAM, while ALCAM did not regulate Gli1/Gli2 protein levels. **A)** A 48 h treatment with GANT61 decreased the protein levels of Gli1, Gli2 and ALCAM in the HN12 cells. The changes in the mRNA level of ALCAM after the 48 h treatment with GANT61 did not achieve a statistically significant difference; **B)** Hedgehog pathway proteins did not change after ALCAM knockdown in the HN12 and HN13 cells. The mRNA level of ALCAM was downregulated after transfection of the shALCAM plasmids into the HN12 and HN13 cells.

the expression levels of Gli1/Gli2 protein did not change (Fig 4B). Over-expression of the ALCAM protein in the HN13 cells did not alter the level of Gli1/Gli2 (Figs 3A and C). In conclusion, ALCAM did not regulate the activity of the SHH signalling pathway.

The mechanism involved in the regulation of the expression level of ALCAM protein by the SHH signalling pathway was investigated.

We ascertained whether the SHH pathway could extend the half-life of ALCAM protein. CHX was used to block the protein synthesis, and SHH recombinant protein was used to activate the pathway. Compared with inactivation of pathway, the activation of this pathway obviously extended the degradation of ALCAM protein (Fig 5A). We next used MG132 to block the degradation of ALCAM. As shown in Figure 5B, the content of ALCAM protein increased with time. In conclusion, the SHH signalling pathway prolonged the ALCAM protein degradation and the degradation of ALCAM protein could pass the ubiquitin-proteasome pathway.

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Discussion

OSCC is a cancer of worldwide concern. Tumour relapse and metastasis are the main reasons for deaths[19]. Therefore, identification of underlying OSCC protein biomarkers predicting tumour metastasis and the elucidation of potential regulatory mechanism affecting biological functions could aid the treatment of OSCC[20].

It brings controversy into existence about the role of ALCAM in cancer development. Sawhney et al showed a significant association between positive cytoplasmic ALCAM expression and clinical stage, tumour size and disease-free survival, but did not observe a relationship between ALCAM expression and nodal status[21]. Van den Brandt et al reported an association between membranous ALCAM expression and nodal metastasis[22]. Clauditz et al found no association between ALCAM positivity and clinical parameters. They also found no significant association between ALCAM expression and survival rates[23]. In the present study, an OSCC tissue array was used to assess ALCAM expression. ALCAM and N stage were independent risk factors for overall survival by multivariate analysis. Patients with over-expression of ALCAM protein had a poor prognosis. This result was different from other previous reports[23,24]. It is possible that the samples in these studies included all HNSCC and led to occasional significant results that did not hold true. We focused on patients with OSCC, and the data fully mirrored the expression features of ALCAM protein in OSCC.

The SHH signalling pathway plays a fundamental role in tumour initiation and/or progression to more advanced tumour stages, embryonic development, maintenance of CSCs and metastasis[8,16,18,25]. Its abnormal activation is closely associated with tumorigenesis, migration, metastasis, cancer stemness and chemo-resistance[9,26]. However, we did not find a close relationship between Glis proteins and survival, which was different from previous reports[13,27].

The reason for the conflictive results is as follows:

- Former literature just focused on Gli1 or Gli2;
- The samples included all HNSCC, which led to an inaccuracy of statistics;
- The small size of the samples included in these studies led to occasional significantly differences. In this study, we excluded the above influences to evaluate the effect of Gli1/ Gli2 in OSCC. Overall survival was not closely correlated with Gli1/ Gli2.

ALCAM protein expression is positively correlated with Gli1/Gli2 protein expression by analysing the correlation between them. The OSCC cell lines also show

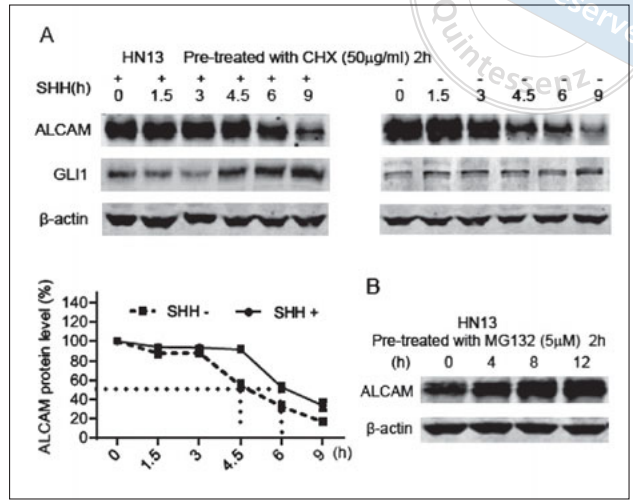

Fig 5 Hedgehog pathway activation prolonged the half-life of ALCAM protein. **A)** Hedgehog pathway activation decreased the rate of ALCAM degradation after HN13 cells were pre-treated with CHX (50 μg/ml) for 2 h. Quantitative analysis of the ALCAM protein level after SHH treatment in the HN13 cells; **B)** MG132 was used to pre-treat the HN13 cells to examine the expression of ALCAM.

these proteins were correlated. SHH also could enhance the invasion and metastasis activity by increasing the expression level of ALCAM in the OSCC cells.

However, the expression level of Gli1/Gli2 protein was not controlled by knocking ALCAM down in cancer cells, which shows the expression level of Gli1/Gli2 was not regulated by ALCAM in the cancer cells. These assays showed that ALCAM protein did not regulate the SHH signalling pathway in OSCC.

To ascertain how the Hedgehog pathway regulates the ALCAM protein, we used CHX to block the protein synthesis, and then used SHH recombinant protein to activate the Hedgehog pathway. The results showed that following stimulation of SHH recombinant protein, the half-life of ALCAM protein was prolonged by 90 min. It indicated the Hedgehog pathway could regulate the expression level of ALCAM by prolonging the half-life of this protein. Other regulatory mechanisms may also be involved in regulating over-expression of ALCAM. In our experiment, Hedgehog pathway in OSCC cell is weakly activated, but the over-expression of ALCAM is relatively high. Future study will discuss this phenomenon and the definite mechanism of ALCAM degradation.

In the present study, we found that the expression of ALCAM protein could serve as a biomarker for poor prognosis in OSCC. The SHH signalling pathway induced invasion and migration activity by prolonging ALCAM half-life in OSCC cells. The ALCAM protein

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degradation is through the ubiquitin-proteasome pathway. The detail mechanisms of how the Hedgehog pathway regulates the degradation of ALCAM protein needs be further investigated and clarified.

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Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Gang CHEN designed the study, analysed the data and prepared the manuscript; Drs Ming YAN and Rong Rong LI designed the study and revised the manuscript; Professor Wan Tao CHEN supervised the study and revised the manuscript.

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