Journal of Cancer Research and Clinical Oncology (2024) 150:398
https://doi.org/10.1007/s00432-024-05922-z

RESEARCH

ROS-mediated ITGB5 promotes tongue squamous cell carcinoma metastasis through epithelial mesenchymal transition and cell adhesion signal pathway

Weixiong Chen[1,3] · Haojie Yang[4] · Lei Huang[1] · Caihong Fang[1] · Limin Yao[1] · Faxin Liu[1] · Tingting Jin[2,3]

Received: 12 March 2024 / Accepted: 12 August 2024 / Published online: 24 August 2024

Abstract

Purpose Integrin β5 (ITGB5) is an integrin β subunit member widely expressed in the human bodies, especially in cancer cells and tissues, which is a key factor in promoting tumor metastasis. In this study we investigated the differential expression of ITGB5 in tongue squamous cell carcinoma (TSCC), especially in those with lymph node metastasis, and revealed the possible mechanism.

Methods The expression of ITGB5 in TSCC was analyzed by database and verified by immunohistochemistry through 135 TSCC patients' tissue sections from Sun Yat-sen Memorial Hospital and Guangzhou First People's Hospital. The relationship between ITGB5 and lymph node metastasis or prognosis was analyzed retrospectively. The effects of ITGB5 on TSCC cells were examined through knocking down or overexpression and its possible regulator and signal pathway were explored.

Results The expression of ITGB5 in TSCC was higher than that in adjacent tissue, and the expression in patients with lymph node metastasis was higher than that in patients without lymph node metastasis. The high expression of ITGB5 predicted a worse prognosis. Knock down of ITGB5 suppressed invasion and migration of TSCC cells, while overexpression of ITGB5 contributed to invasion and migration. Reactive oxygen species (ROS) regulated epithelial mesenchymal transition (EMT), and we further verified that ROS enhanced the expression of ITGB5 to promote the metastasis of TSCC. Mechanistically, ITGB5 functions through cell adhesion signal pathway.

Conclusion The increased expression of ITGB5 in tongue squamous cell carcinoma with lymph node metastasis may be a potential target for evaluating lymph node metastasis and worse prognosis of tongue squamous cell carcinoma. Scavenge of ROS or knock down of ITGB5 may be the strategies to overcome metastasis of TSCC.

Keywords ITGB5 · Tongue squamous cell carcinoma · Lymph node metastasis · Reactive oxygen species · Epithelial mesenchymal transition

Weixiong Chen and Haojie Yang contributed equally to this work.

✉ Weixiong Chen
453797451@qq.com

✉ Tingting Jin
649853763@qq.com

[1] Department of Stomatology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou 510180, PR China

[2] Department of Oral and Maxillofacial Surgery, Stomatological Hospital, Southern Medical University, Guangzhou 510115, PR China

[3] Department of Stomatology, Longgang District Central Hospital, Chinese University of Hong Kong, Shenzhen 518116, PR China

[4] Department of Anesthesiology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, PR China

Abbreviations

ITGB5	Integrin β5
TSCC	Tongue squamous cell carcinoma
ROS	Reactive oxygen species
EMT	Epithelial mesenchymal transition
TGF-β	Transforming growth factor-β
GEPIA	Gene Expression Profiling Interactive Analysis
RIPA	Radioimmunoprecipitation assay
HNSC	Head and neck squamous cell carcinoma
FAK	kinase focal adhesion kinase

Introduction

Tongue squamous cell carcinoma (TSCC) is the most common type of oral cancer, with a five-year survival rate of less than 50% and a high recurrence and metastasis rate. Due to the high mechanical activity and abundant blood supply of the tongue, the risk of metastasis is increased, and mainly lymph node metastasis occurred (Fang et al. 2019; Liu et al. 2020). Therefore, exploring individualized therapies based on molecular targets is particularly important for suppressing lymph node metastasis of TSCC. Currently, tumor size, lymph node metastasis, histopathological grade, and serum cancer markers can be used as prognostic evaluation factors for TSCC patients (Al-Jamaei AAH, et al. 2023). Lymph node metastasis is closely related to the prognosis of tongue squamous cell carcinoma, and is the most commonly used clinical prognostic evaluation factor (Zhuge et al. 2023). However, it cannot be applied in the early stages before metastasis. In addition, it is too late to make any judgment and obtain the best treatment after the occurrence of metastasis. Establishing biomarkers that reflect biological functions will enable clinicians to accurately diagnose cancers at an early stage and optimize treatment.

The integrin family is a transmembrane glycoprotein widely present in cell membranes, composed of α and β. So far, eight members of the integrinβ (ITGB) proteins have been identified in living organisms (Chen et al. 2022). Previous studies have unrevealed the carcinogenic effects of deregulated integrin signaling in several malignant tumors (Gu et al. 2023; Zhang et al. 2022; Choudhury et al. 2023), but the prognostic value and potential biological functions of the ITGB proteins in TSCC are still largely elusive. The ITGB proteins Integrin β 5 (ITGB5) is an integrin β subfamily consisting of extracellular binding regions, transmembrane regions, and intracellular regions, with spatially N-terminal, C-terminal, repetitive sequences, and specific domains [Setti et al. 2013]. Multiple studies have shown that ITGB5 promotes the migration and invasion of cancer cells, and it plays a key role in TGF- β induced epithelial-mesenchymal transformation (EMT) (Hirano et al. 2020; Shi et al. 2021). In glioblastoma, elevated ITGB5 expression was highly correlated with glioma progression and was required for migration and invasion of glioma cells through EMT (Zhang et al. 2019). However, the relationship between ITGB5 and tumor metastasis and prognosis in TSCC is unclear.

This study examined the expression of ITGB proteins in TSCC patients. Importantly, ITGB5 is the most upregulated protein in TSCC patients, so we further examined its expression and analyzed its relationship with lymph node metastasis and prognosis. Furthermore, we revealed the underlying mechanism that ROS may enhance the expression of ITGB5, which promoted the metastasis of TSCC through epithelial-mesenchymal transformation. The in vitro and in vivo effects of ITGB5 proved its prognostic value in TSCC.

Materials and methods

TSCC patient cohort

32 pairs of tumor tissues and adjacent paracancerous tissues were obtained from the TSCC patients. We employed the qRT-PCR method for comparing the mRNA expression of ITGB1-7 in TSCC patients' samples. RNA was extracted from TSCC patient's fresh dissected tissues using the RNA Quick Purification kit (ES science, Cat#RN001). qRT-PCR was performed using SYBR Green Real-time PCR Master Mix (ReverTra Ace, Toyobo) and a LightCycler 480 (Roche, Basel, Switzerland). Based on the obtained Ct values, we compared the expression of ITGB1-7 in TSCC tumor tissue as the relative expression levels in paracancerous tissues were set to 1. A total of 103 cases of TSCC and its adjacent tissues were collected from the Department of Oral and Maxillofacial Surgery of Sun Yat-sen University's Sun Yat-sen Memorial Hospital (83 cases) and the Department of Stomatology of Guangzhou First People Hospital (20 cases) from June 2008 to October 2015 to perform a retrospective analysis.

Inclusion criteria is as following: All patients were primary TSCC patients. Tissues were diagnosed as tongue squamous cell carcinoma by pathological biopsy according to 8th edition of AJCC Staging System. No distant organ metastasis was found in all patients. All enrolled patients did not receive any other anti-tumor treatment before surgery. Exclusion criteria is as following: Patients with recurrent tongue squamous cell carcinoma. Patient received chemotherapy, radiotherapy or other non-surgical treatment before surgery.

The removed tissue specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and 4 μm -thick tissue sections were prepared for following study. All patients have been informed and signed an accurate informed consent form approved by the Ethics Committee of the two

hospitals, and all the experiments were performed in accordance with approved guidelines and regulations.

GEPIA database analysis

By analyzing the expression level of ITGB5 gene in head and neck squamous cell carcinoma through the GEPIA database (http://gepia.cancer-pku.cn/) (Tang et al. 2017). Gene Expression Profile was used to analyze the expression of ITGB5 in the cancer and adjacent tissues of head and neck squamous cell carcinoma.

Immunohistochemistry

The 4 μm tissue sections of tongue squamous cell carcinoma were dewaxed with xylene, hydrated with gradient ethanol (95%, 90%, 85%, 75%), and then repaired with antigen by microwave oven. After that, tissue sections were incubated at room temperature with 3% hydrogen peroxide for 10 min to remove endogenous peroxidase. Next, tissue sections were permeated with PBS solution containing 0.1% Triton X-100 for 5 min, washed with PBS, added 5% goat serum, and sealed at room temperature for 1 h. Excess liquid was tossed off from the tissue sections, ITGB5 antibody (abcepta, Cat#AP14000a) were added and incubated in a refrigerator at 4 ℃ overnight. On the second day, the first antibody solution of the tissue sections was removed, washed with PBS, and then the pika universal second antibody was added dropwise to incubate at room temperature for 30 min. DAB was colored under microscope for 3–5 min, and hematoxylin was re-stained for 1–2 min. Dehydrated, transparent, neutral resin seal, observed under a microscope. Read 1000 cells per tissue section, and if the number of positive cells is greater than 350, it indicates high expression.

Cell culture

Cal27 and Scc9 were purchased from the American Type Culture Collection. cisplatin (Sigma, St. Louis, MO, USA) at concentrations of 10^{-7} M to 10^{-5} M were used to treat Cal27 or Scc9 to establish the stable cisplatin-resistant lines Cal27-re and Scc9-re. Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (ABW) were supplied to cultivate Cal27 and Cal27-re cells. Dulbecco's modified Eagle's medium-F12 (Gibco) supplemented with 10% fetal bovine serum (ABW) were supplied to cultivate Scc9 and Scc9-re cells.

Transfections

The shRNA was purchased from TranSheep Bio. The various targeting sequences were as follows: sh-ITGB5, 5-CCCGCTATGAAATGGCTTCAA-3. ITGB5 cDNA was purchased from TranSheep Bio. To generate lentivirus expressing sh-ITGB5 or ITGB5, HEK 293T cells grown on a T75 dish were transfected with 15 μg pCDH-sh-ITGB5 or pCDH-ITGB5 or control vector (pCDH), 10 μg psPAX2, and 5 μg pMD2G. 6 h after the transfection, cells were cultured with DMEM containing 10% FBS. Virus supernatant was collected twice at 48 and 72 h after transfection with a 0.45 μm Filter and centrifuged in a 40 mL ultracentrifugation tube at 4 ℃ for 120 min at 72,000 g/min. Resuspended lentivirus using PBS. Then lentivirus along with polybrene were used to transfect TSCC cells. Puromycin (1 ug/mL) was used as a selection marker for the infected cells. The expression efficiency was evaluated by qRT-PCR and Western blot.

RNA and protein extraction

For RNA extraction, total RNA was extracted by the RNA Quick Purification kit (ES science, Cat#RN001), according to the instructions of the manufacturer. For protein extraction, the cells were rinsed three times with PBS for 5 min each, followed by the addition of radioimmunoprecipitation assay (RIPA) Buffer (Kangweishiji, Cat# CW2333S) containing a protease inhibitor cocktail (APExBIO, Cat# K1007). The cells were lysed for 0.5 h at 4 °C. Then, the samples were scraped down and centrifuged at a speed of 12,000 rpm for 20 min at 4 °C. The supernatants were collected as the total protein.

qRT-PCR

qRT-PCR was performed using SYBR Green Real-time PCR Master Mix (ReverTra Ace, Toyobo) and a LightCycler 480 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Reactions were performed in triplicate in three independent experiments. The sequences of all primers were listed in sup Table 1. The relative expression levels in the control were set to 1.

Western blot

Protein extracts were resolved via 8% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (BioRad, Berkeley, CA, USA), probed with antibodies against human ITGB5 (abcepta, Cat#AP14000a), GAPDH (HUABIO, Cat#HA1031), E-cadherin (Immunoway, Cat#YT1454), Vimentin (Immunoway, Cat#YT4880) and then with a peroxidase-conjugated secondary antibody (Beijing Ruikang); the bands were visualized via chemiluminescence (BIO-RAD, ChemiDoc^{TM}Imaging System).

Table 1 Correlation between ITGB5 and clinicopathological status in TSCC patients

Characteristics	Number of cases	ITGB5 expression (%)		χ^2	*P*
		No. of low expression	No. of high expression		
Sex					
Male	55	31 (56.36)	24 (43.67)	1.631	0.202
Female	48	21 (43.75)	27 (56.25)		
Age					
< 50岁	37	15 (40.54)	22 (59.46)	2.285	0.131
≥ 50	66	37 (56.06)	29 (43.94)		
T stage					
T1-2	58	28 (48.28)	30 (51.72)	0.259	0.611
T3-4	45	24 (53.33)	21 (46.67)		
Node metastasis					
N0	61	40 (65.57)	21 (34.43)	13.624	< 0.001
N+	42	12 (28.57)	30 (71.43)		
Clinical stages					
I ~ II	55	29 (52.73)	26 (47.27)	0.237	0.626
III ~ IV	48	23 (47.92)	25 (52.08)		
Status					
Survival	60	40 (66.67)	20 (33.33)	15.054	< 0.001
Death	43	12 (27.91)	31 (72.09)		

Modified boyden chamber assay

In all, 1×10^5 TSCC cells were plated into the upper chamber of a polycarbonate transwell filter chamber (Corning, NewYork, NY, USA) and incubated for 22 h. For invasion assay, the upper chamber was coated with Basement Membrane (R&D, Minneapolis, MN, USA). Cells on the lower membrane surface were fixed in 4% paraformaldehyde, stained with crystal violet and counted (5 random 100× fields per well). Three independent experiments were performed and the data are presented as the average ± s.d.

ROS production assay

The intracellular ROS levels were measured by detecting the conversion of cell-permeable 2,7-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) (Beyotime Biotechnology, S0033). The cells were seeded in 6-well plates and treated under different conditions. Then, all wells were washed with PBS three times, and the cells were incubated with DCFH-DA at 37 °C for 25 min. The cells were washed with serum-free culture medium. Then the cells were fixed with 4% paraformaldehyde and DAPI staining. Finally, the DCF fluorescence distribution was detected by a confocal microscopy (ZEISS, LSM900).

mRNA array and KEGG

mRNA array analyses were compared between Cal27 with or without ITGB overexpression. The differentially expressed mRNAs and KEGG enrichment were calculated, identified, and visualized (KangchengBio Corporation, Shanghai, China).

Xenografts

The animal experiments were approved by the Sun Yat-sen University Laboratory Animal Care and Use Committee. Male BALB/c-nu mice (4–6 weeks old) were randomly divided into 4 groups ($n = 6$) and applied to evaluate the effects of ITGB5 in TSCC cells in vivo. TSCC cells with ITGB5 knock down or overexpressed were transfected with luciferase reporter plasmid (OBiO Technology, Shanghai). 1×10^6 cells

in 200 mL of PBS were injected into the mice via the tail vein. The mice were imaged 1.5 months later with luciferase-based IVIS Lumina Imaging System (Xenogen, Alameda, CA, USA) after injection of 100 mL of D-luciferin (15 mg/mL) (Yeasen, Shanghai) 10 min before imaging. After imaging, lungs were harvested from euthanized mice and fixed in paraformaldehyde for immunohistochemistry.

Statistical analysis

SPSS 25.0 statistical software was used to analyze and process the data. Chi-square test was used to compare the inter group rates. Student's t test was used to compare the mean between the two groups to analyze the correlation between the expression of ITGB5 and the clinical status of patients. The Kaplan Meier was used to analyze the survival of patients. $P < 0.05$ indicates statistical significance.

Results

High expression of ITGB5 predicted a worse survival outcome in TSCC patients

Firstly, we examined the ITGB proteins in fresh dissected TSCC tissues by qRT- PCR. Among eight proteins, ITGB1, ITGB5, ITGB7 and ITGB8 showed higher expression in tumors than that in the normal adjacent tissues, and ITGB5 showed the highest one, so we focused on the function of ITGB5 in the following study. GEPIA database organically integrates TCGA cancer data and GTEx normal tissue data, which is conducive to mining new cancer targets and markers. Through searching, we found that the expression of ITGB5 was higher in head and neck squamous cell carcinoma (HNSC) than in adjacent tissues (Fig. 1A, B). In order to further clarify the expression of ITGB5 in TSCC, we performed immunohistochemistry on tissue samples from TSCC patients. Compared to the negative control (Fig. 1C1), we found that ITGB5 was mainly

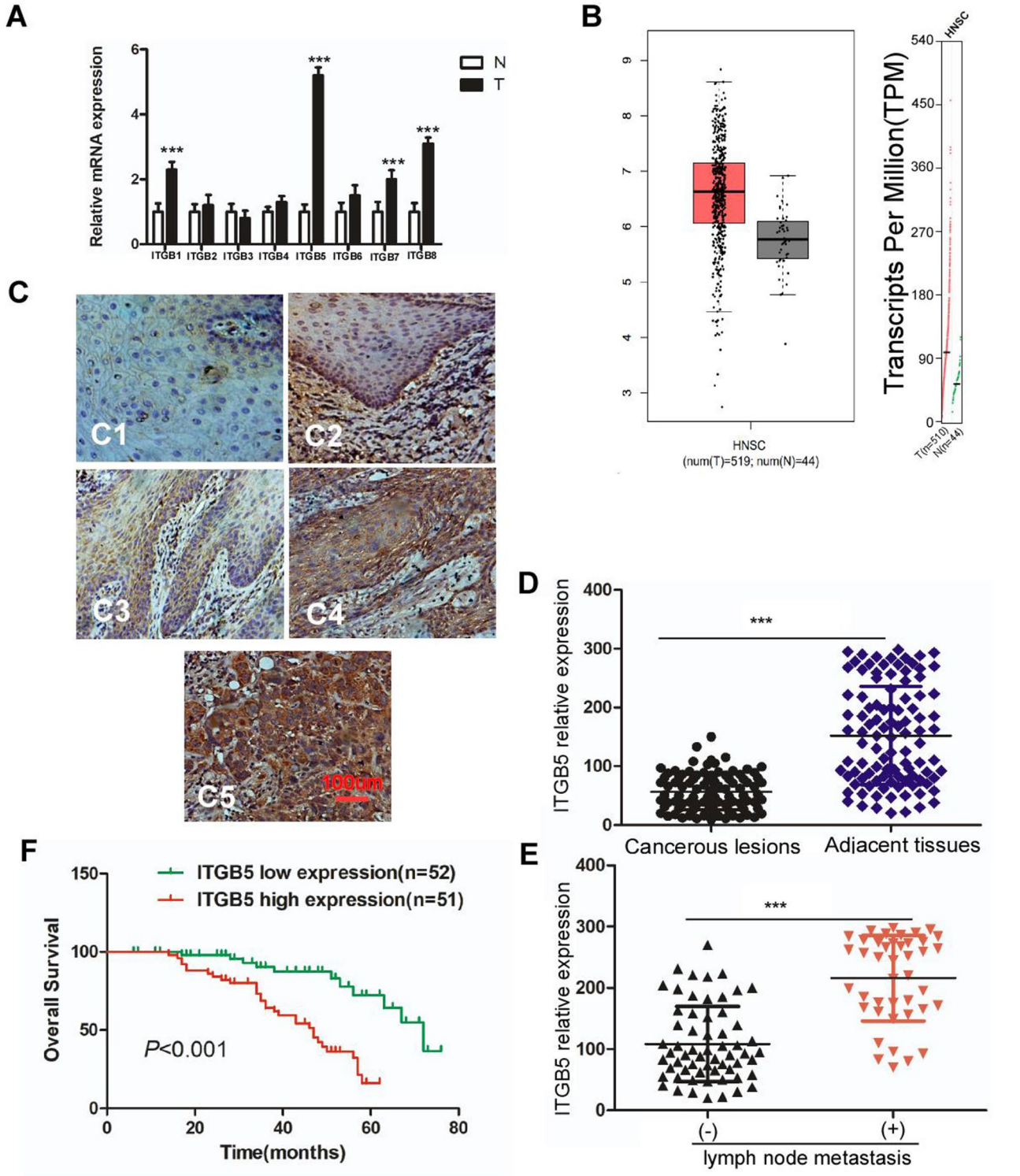

Fig. 1 Expression of ITGB5 and prognosis in TSCC patients. **A** mRNA expression of ITGB1-7 in TSCC and adjacent tissues. ***$P < 0.001$. **B** Expression of ITGB5 in HNSC and adjacent tissues. Copy number of ITGB5 in HNSC. **C** Immunohistochemistry showed the expression of ITGB5 in normal tissues and TSCC tissues (× 200), **C1** Negative control, **C2** Normal tissue, **C3** Paracancerous tissue, **C4** Primary TSCC without lymph node metastasis, **C5** Primary TSCC with lymph node metastasis. **D** The relative expression of ITGB5 in the cancerous lesions and the adjacent tissues. ***$P < 0.001$.**E** The relative expression of ITGB5 in TSCC with and without lymph node metastasis. ***$P < 0.001$.**F** Analysis of the expression of ITGB5 and the survival curve of 103 TSCC patients

expressed in the cytoplasm, and its expression level was extremely low in normal tissues (Fig. 1C2), while it was weakly expressed in adjacent tissue (Fig. 1C3). In TSCC, the expression of ITGB5 in the cancerous lesions significantly higher than those in the adjacent tissues (Fig. 1C4, D), and in patients with lymph node metastasis, the expression was higher than in patients without lymph node metastasis (Fig. 1C5, E). The above results suggested that ITGB5 was closely related to the malignant degree of TSCC, and was significantly related to lymph node metastasis. We analyzed the expression of ITGB5 in 61 TSCC patients without lymph node metastasis and 42 TSCC patients with lymph node metastasis. Clinical retrospective analysis indicated that high expression of ITGB5 was closely correlated with lymph node metastasis in TSCC patients ($P < 0.001$), while the expression level of ITGB5 was not significantly correlated with sex, age, tumor size, clinical stage of the patient ($P > 0.05$). Moreover, high expression of ITGB5 predicted a worse prognosis for the patient. The results were shown in Table 1. We analyzed the relationship between the expression of ITGB5 and the prognosis of TSCC patients by Kaplan Meier, and found that the median survival time of TSCC patients in the low expression group of ITGB5 was 72 months, while the median survival time of TSCC patients in the high expression group of ITGB5 was 47 months, with a statistically significant difference ($P < 0.001$) (Fig. 1F).

ITGB5 contributed to the invasion and migration of TSCC cells through EMT

Our previous study showed that chemo-resistant TSCC cells had more invasive ability (Lin et al. 2018). We hypothesized that ITGB5 may also involve in the invasive behavior in chemotherapy induced metastasis. qRT-PCR indicated that ITGB5 showed up-regulation in two pairs chemo-resistant TSCC cells relative to parental TSCC cells, and Western blots further proved this expression (Fig. 2A, B). We used shRNA to attenuate the expression of ITGB5 (Fig. S1A and Fig. 2C), and found that down-regulation of ITGB5 could inhibit the invasion and migration of chemo-resistant TSCC cells (Fig. 2D). Chemo-resistant TSCC cells had experienced EMT, and down-regulation of ITGB5 could reverse EMT (Fig. 2E). We used lentivirus to overexpress ITGB5 in parental TSCC cells Cal27 and Scc9 (Fig. S1B and Fig. 2F). Overexpression of ITGB5 enhanced the invasion and migration of parental TSCC cells and promoted EMT (Fig. 2G, H). Taken together, these results suggested that ITGB5 promoted the invasion and migration of TSCC cells through EMT.

ROS enhanced the expression of ITGB5 to promote the metastasis of TSCC through EMT

We previously showed that chemo-resistant TSCC cells had more reactive oxygen species (ROS) (Fan et al. 2019). The effects of ROS on cellular responses including signaling and transcription have been attributed to the oxidative modification of proteins, nucleic acids and lipids, so we supposed that ROS may regulate the expression of ITGB5 to affect EMT. We used NAC, a ROS scavenger to decrease the cellular level of ROS in chemo-resistant TSCC cells (Fig. 3A). Decrease of ROS attenuated the invasion and migration of chemo-resistant TSCC cells (Fig. 3B). Intriguingly, scavenge of ROS decreased the expression of ITGB5 and reversed EMT of TSCC cells (Fig. 3C, D). When we supplied parental TSCC cells with H_2O_2 (Fig. 3E), the activities of invasion and migration were enhanced (Fig. 3F), similar to previous report (Lu et al. 2019). ITGB5 were up regulated with H_2O_2 supplement, and EMT was induced in parental TSCC cells (Fig. 3G, H). Furthermore, we performed rescue assay to confirmed the relationship between ROS and ITGB5. In ITGB5 knockdown TSCC cells, H_2O_2 supplement could enhance the expression of ITGB5, while NAC can decrease the expression of ITGB5-over expressed TSCC cells, which confirmed that ROS was the regulator of ITGB5 (Fig. 3I). In summary, ROS up-regulated the expression of ITGB5 to promote metastasis of TSCC through EMT.

ITGB5 functions in cancer and cell adhesion related pathways in TSCC

To study the mechanism by which ROS -ITGB5 promoted metastasis, we used mRNA microarray to study Cal27 cells with or without ITGB5 overexpression (Sup Table 2). mRNA microarray indicated that the DEGs were enriched in signal pathway associated with tissue invasion and metastasis, such as cell adhesion molecules, focal adhesion (Fig. 4A and B, Sup Table 3). qRT-PCR verified that the expression of several cell adhesion molecules were increased with ITGB5 overexpression, such as CDH2, CDH4, CDH5, CLDN23, ICAM1, OCLN (Fig. 4C). These results indicated that ITGB5 functions through cell adhesion signal pathway.

ITGB5 contributed to TSCC metastasis in vivo

To explore the effect of ITGB5 on TSCC cells in vivo, We performed xenograft in a nude mice model. ITGB5 overexpressed Cal27 or ITGB5 knockdown Cal27-re were injected into nude mice via tail vein. The ITGB5 overexpression group had more and larger liver lung metastases,

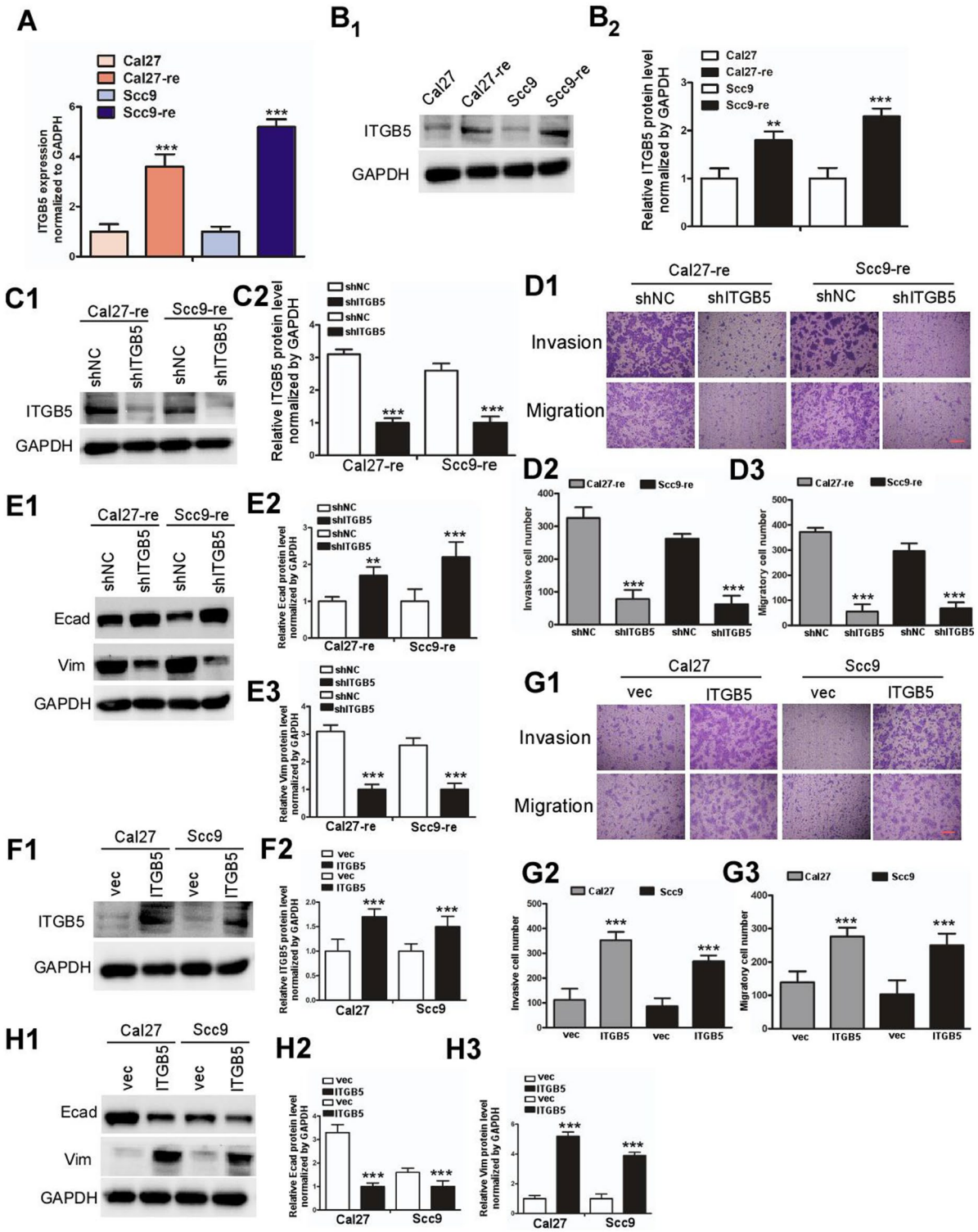
A
Cal27
Cal27-re
Scc9
Scc9-re
ITGB5 expression normalized to GADPH
B1
Cal27
Cal27-re
Scc9
Scc9-re
ITGB5
GAPDH
B2
Relative ITGB5 protein level normalized by GAPDH
C1
Cal27-re
Scc9-re
shNC
shITGB5
ITGB5
GAPDH
C2
Relative ITGB5 protein level normalized by GAPDH
D1
Invasion
Migration
D2
Invasive cell number
D3
Migratory cell number
E1
Ecad
Vim
E2
Relative Ecad protein level normalized by GAPDH
E3
Relative Vim protein level normalized by GAPDH
F1
vec
ITGB5
F2
Relative ITGB5 protein level normalized by GAPDH
G1
G2
G3
H1
H2
H3

Fig. 2 ITGB5 enhanced the invasion and migration of TSCC cells through EMT. **A** qRT-PCR showed the RNA level of ITGB5 in two pair of TSCC cells, Cal27 and Cal27-re, Scc9 and Scc9-re. ***$P<0.001$. **B** Representative Western blots and quantitative data showed the protein level of ITGB5 in two pair of TSCC cells, Cal27 and Cal27-re, Scc9 and Scc9-re. **$P<0.01$. ***$P<0.001$. **C** Representative Western blots and quantitative data showed the protein level of after knocking down ITGB5 with shRNA in chemo-resistant TSCC cells. ***$P<0.001$. **D** The representative images of Transwell assays (**D1**) and the data analysis results (**D2–D3**) showed that knockdown of ITGB5 attenuated the migration and invasion of chemo-resistant TSCC cells. The invaded or migrated cells were detected 22 h after cells were seeded. Scale bars = 200 μm. ***$P<0.001$. **E** Representative Western blots and quantitative data showed the expression of EMT markers, Ecadherin (Ecad) and Vimentin (Vim) after knocking down ITGB5 in chemo-resistant TSCC cells. ***$P<0.001$. **F** Representative Western blots and quantitative data showed the protein level of ITGB5 after overexpressing ITGB5 in parental TSCC cells. ***$P<0.001$. **G** The representative images of Transwell assays (**G1**) and the data analysis results (**G2–G3**) showed that overexpressing ITGB5 contributed to the migration and invasion of parental TSCC cells. The invaded or migrated cells were detected 22 h after cells were seeded. Scale bars = 200 μm. ***$P<0.001$.**H** Representative Western blots and quantitative data showed the expression of EMT markers, Ecadherin (Ecad) and Vimentin (Vim) after overexpressing ITGB5

while knocking down ITGB5 resulted in a reduction in lung metastasis (Fig. 5A), which was further confirmed by HE staining of lung tissue tissue sections (Fig. 5B). We detected ITGB5 in each group through immunohistochemistry to verify the expression of ITGB5 (Fig. 5C). These results further proved that ITGB5 contributed to TSCC metastasis in vivo.

Discussion

Metastasis is still a major difficulty in curing cancer in clinical practice even though surgical and non-surgical treatments are fully utilized (Lambert et al. 2017). TSCC, as the most common type of oral cancer, is prone to metastasis to lymph nodes, which predicts a worse prognosis (Fang et al. 2019; Liu et al. 2020). Therefore, excavating molecular targets is particularly important for inhibiting lymph node metastasis in TSCC. The first step in tumor metastasis is to break through various cellular barriers, and integrins play an important role in this process (Shen et al. 2019). Integrins are composed of 18 α- Subunits and 8β- Heterodimeric cell surface receptors, which regulate a variety of biological functions in cancer, including proliferation, adhesion, migration and invasion. More importantly, integrins' interaction with oncogenes makes them potential targets for tumor therapy (Cooper et al. 2019). In our study, we examined 8 ITGB protein in TSCC patients and found ITGB5 was the mostly up-regulated one, and the database analysis and retrospective analysis from two hospitals further proved ITGB5 may predict the TSCC metastasis and a worse survival outcome.

ITGB5 is only related with ITGαV, and accumulating evidence have shown that it is related to various tumor pathological conditions (Slack et al. 2022). The critical role of some integrin has been suggested in TSCC (Bianchi et al. 2010; Lv et al. 2020; Stojanovic et al. 2016). Increasing evidence supported the role of ITGB5 in promoting cancer cell migration, invasion, and transforming growth factors β (TGF-β) key roles in induced EMT (Zhang et al. 2019; Bianchi et al. 2010). Wei Shi et al. found that ITGB5 expression was increased in colorectal cancer (CRC) and was significantly correlated with the progression of CRC and poor overall survival in CRC patients. Silencing ITGB5 significantly inhibited the invasion of human CRC cells, and inhibited the metastasis of CRC tumors in vivo (Shi et al. 2021). ITGB5 was enriched in the exosomes of liver metastatic pancreatic cancer, which indicated its potential role in tumor progression and metastasis (Wortzel et al. 2019). Multiple studies have revealed that ITGB5 was overexpressed in gliomas, especially in mesenchymal subtypes, and that high ITGB5 levels are independently associated with a shorter survival time of patients (Zhang et al. 2019; Denadai et al. 2013). Research has showed that ITGB5 had a significant role in promoting lymph node metastasis in colorectal adenocarcinoma (Denadai et al. 2013). There are few studies on the function of ITGB5 in oral cancer, especially on the role of ITGB5 in TSCC metastasis. Akira Kurokawa et al. found that the expression levels of ITGA3, ITGB4, and ITGB5 could be chosen as candidate biomarkers for cervical lymph node metastasis or TSCC death outcomes, but they only used real-time polymerase chain reaction to detect ITGB5 (Kurokawa et al. 2008). Differently, our in virto and in vivo results revealed that ITGB5 had powerful function in promoting TSCC metastasis. We used siRNA or Lentivirus high expression vector to regulate the expression of ITGB5 in TSCC and showed that ITGB5 could enhance TSCC metastasis. Further animal results confirmed the effect of ITGB5 in promoting TSCC metastasis. In summary, we provided solid evidence that ITGB5 may contribute to TSCC metastasis.

EMT is a classical theory for revealing cancer metastasis. Cancer cells that develop EMT are characterized by increased motility and aggressiveness, favoring their distant spread and metastasis (Pastushenko et al. 2019). Previous studies have demonstrated aberrant expression of ITGB5 regulated metastasis through EMT (Hirano et al. 2020; Shi et al. 2021). Similarily, we showed that ITGB5 promoted the invasion and migration of TSCC cells through EMT. Our previous study showed that chemo-resistant TSCC cells had more invasive ability, namely underwent epithelial-mesenchymal transition (Lin et al. 2018). Intriguingly, ITGB5 promoted TSCC metastasis through EMT. Upregulation of ITGB5 can increased EMT, while downregulation

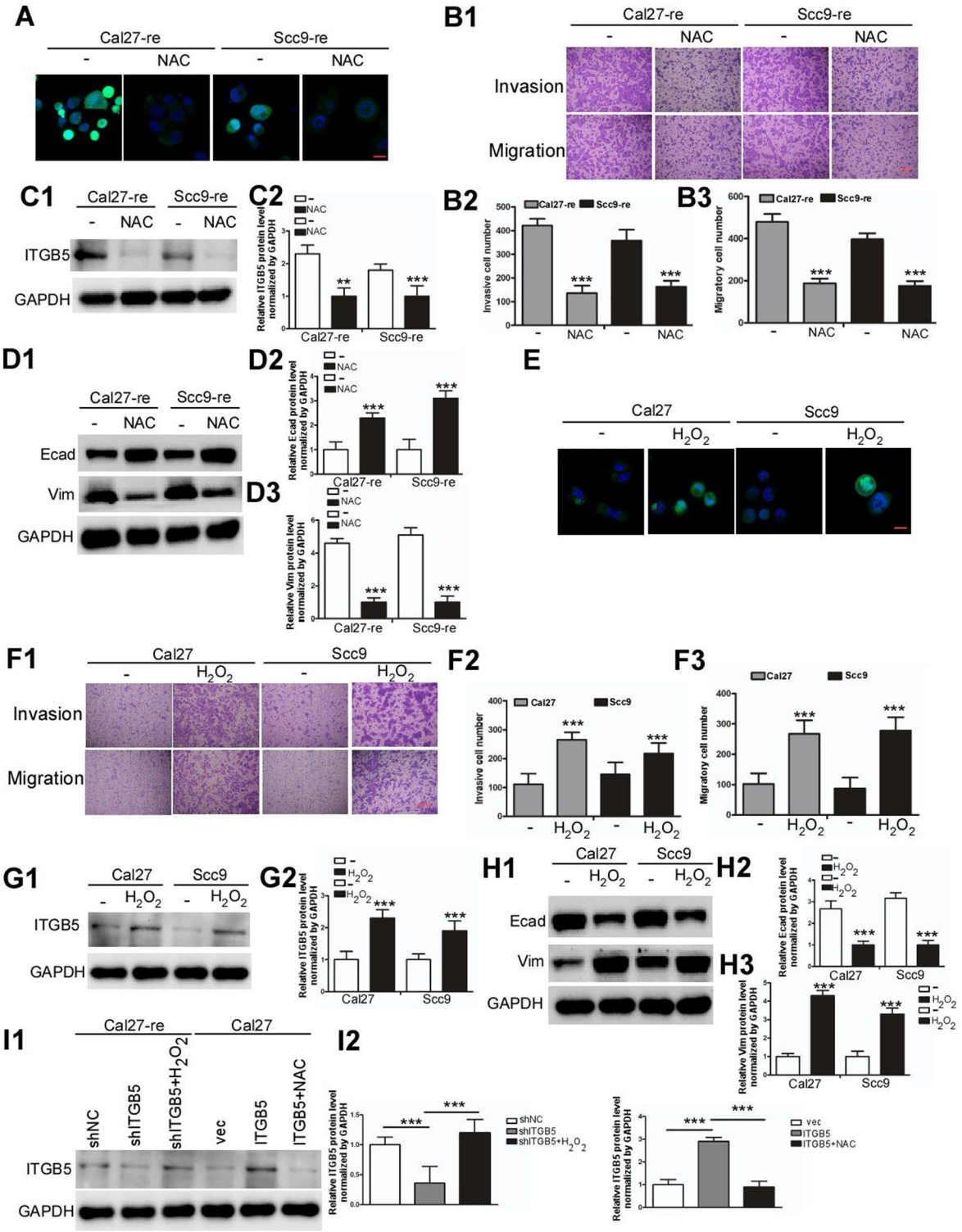
A
Cal27-re
Scc9-re
-
NAC
B1
Invasion
Migration
C1
ITGB5
GAPDH
C2
Relative ITGB5 protein level normalized by GAPDH
B2
Invasive cell number
B3
Migratory cell number
D1
Ecad
Vim
D2
Relative Ecad protein level normalized by GAPDH
D3
Relative Vim protein level normalized by GAPDH
E
Cal27
Scc9
H2O2
F1
F2
F3
G1
G2
H1
H2
H3
I1
shNC
shITGB5
shITGB5+H2O2
vec
ITGB5
ITGB5+NAC
I2
shITGB5+H2O2
ITGB5+NAC

Fig. 3 ROS regulated the expression of ITGB5 to promote the metastasis of TSCC through EMT. **A** DCFH-DA probe indicated the ROS generation by NAC treatment (100 μm, 24 h) in chemo-resistant TSCC cells, Scale bars = 10 μm. **B** The representative images of Transwell assays (**B1**) and the data analysis results (**B2–B3**) showed that NAC attenuated the migration and invasion of chemo-resistant TSCC cells. The invaded or migrated cells were detected 22 h after cells were seeded. Scale bars = 200 μm. ***$P < 0.001$.**C** Representative Western blots and quantitative data showed the expression of ITGB5 after NAC treatment in chemo-resistant TSCC cells. **$P < 0.01$. ***$P < 0.001$. **D** Representative Western blots and quantitative data showed the expression of EMT markers, Ecadherin (Ecad) and Vimentin (Vim) after NAC treatment in chemo-resistant TSCC cells. ***$P < 0.001$. **E** DCFH-DA probe indicated the ROS generation by H_2O_2 treatment (100 mM, 48 h) in parental TSCC cells, Scale bars = 10 μm. **F** The representative images of Transwell assays (**F1**) and the data analysis results (**F2–F3**) H_2O_2 enhanced the migration and invasion of parental TSCC cells. The invaded or migrated cells were detected 22 h after cells were seeded. Scale bars = 200 μm. ***$P < 0.001$. **G** Representative Western blots and quantitative data showed the expression of ITGB5 after H_2O_2 treatment in parental TSCC cells. ***$P < 0.001$. **H** Representative Western blots and quantitative data showed the expression of EMT markers, Ecadherin (Ecad) and Vimentin (Vim) after H_2O_2 treatment in parental TSCC cells. ***$P < 0.001$.**I** Representative Western blots and quantitative data showed the expression of ITGB5 in rescue assay

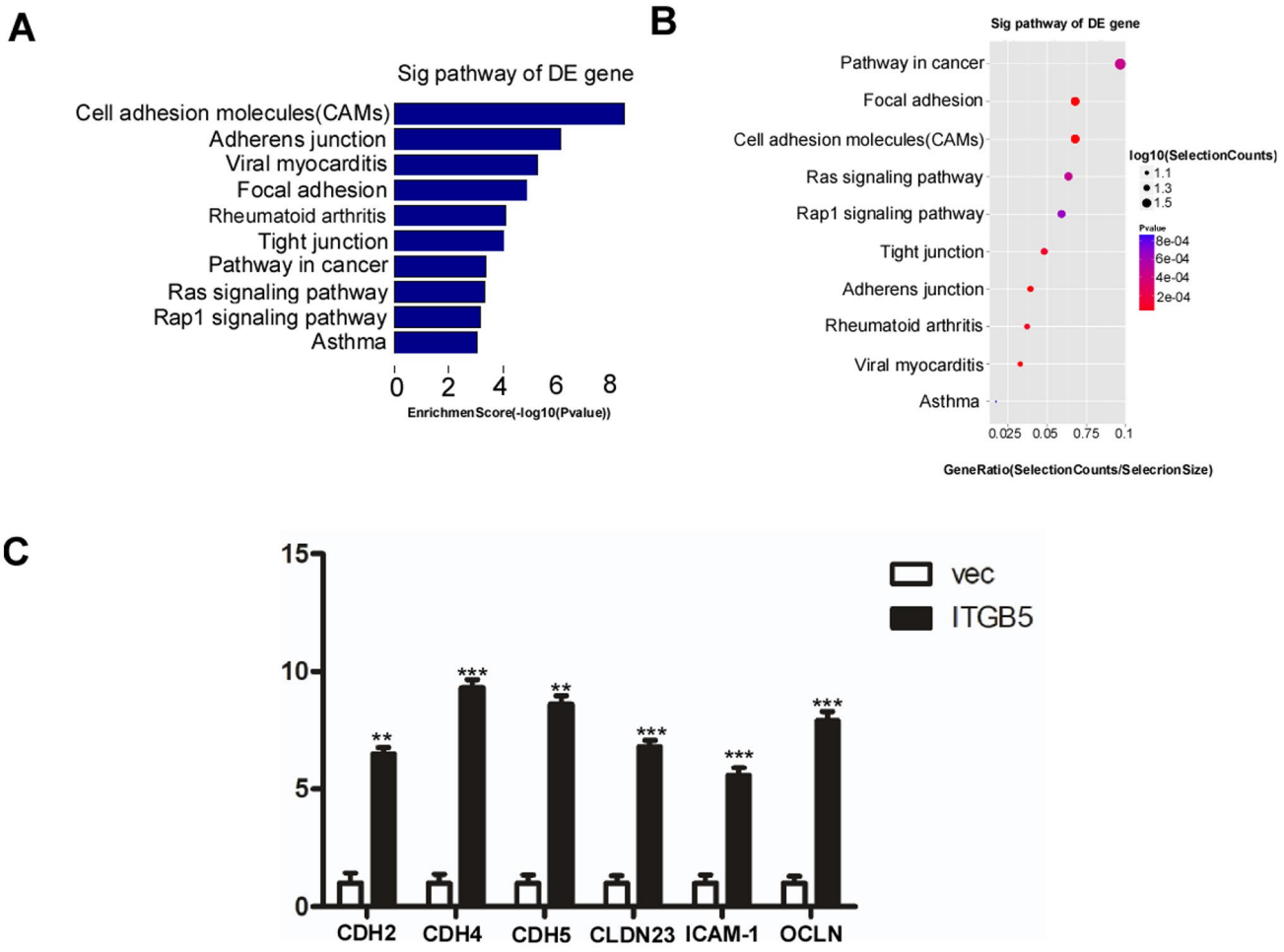

Fig. 4 KEGG analysis of the mechanism by which ITGB5 promoted tumor metastasis. **A**, **B** KEGG enrichment. **C** mRNA expression of CDH2, CDH4, CDH5, CLDN23, ICAM1, OCLN in parental TSCC cells without or with ITGB5 overexpression. **$P < 0.01$. ***$P < 0.001$

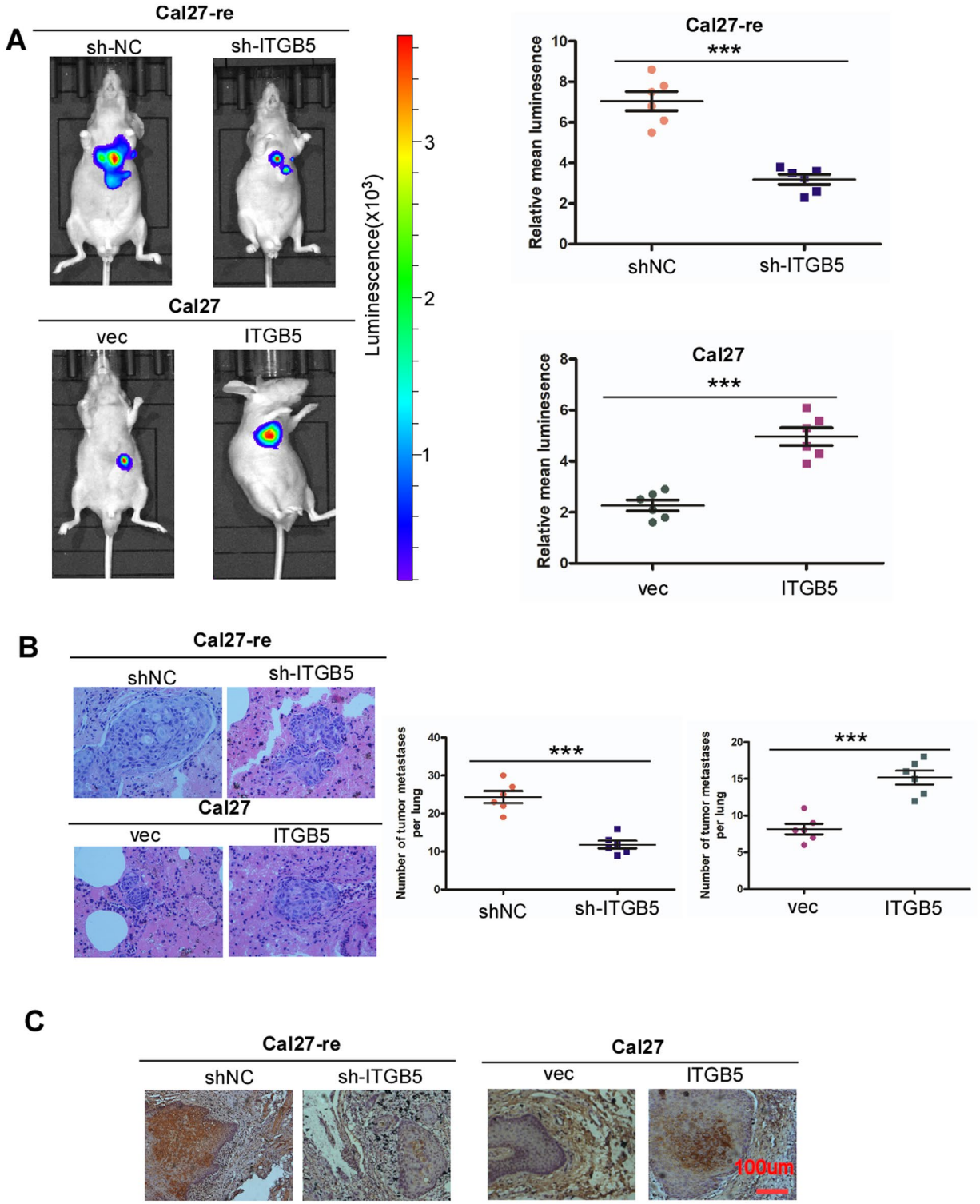

Fig. 5 **A** The representative images and quantitative data showed the bioluminescence signal intensity of each nude mouse after knocking down ITGB5 in chemo-resistant TSCC cells or overexpressing ITGB5 in parental TSCC cells, ***$P < 0.001$. **B** Representative images of HE staining of metastatic tumors in nude mouse lungs, quantitative data indicated the number of metastases, ***$P < 0.001$. **C** Detection of ITGB5 by immunohistochemistry

of ITGB5 may reverse EMT. But the underlaying mechanism of ITGB5 function through is unclear in TSCC metastasis. In terms of mechanism, Huang et al. found that c-Met binded to KRT16 through ITGB5 to form a complex that mediates c-Met/ITGB5 related signal transduction in oral squamous cell carcinoma cells (Huang et al. 2019). This study revealed that KRT16 is associated with ITGB5 and c-Met to promote metastasis in OSCC cells, but didn't emphasize the clinical role of ITGB5 in the regulation of TSCC metastasis (Huang et al. 2019). Different to former reports, we revealed that ROS-mediated ITGB5 expression to affect EMT and the invasion and migration of TSCC cells. Our study shielded light that anti-ROS may be a supplementary method to overcome TSCC metastasis, and some small molecule compounds such as ROS scavenger NAC may have clinical prospect in treating metastatic TSCC.

Signal pathway dysregulation can also provide insights for treatment. In breast cancer, ITGB5 promoted matrix adhesion and signal transduction through Src FAK and MEK-ERK pathways that operate independently in tumor cells, thereby promoting tumor growth and angiogenesis (Bianchi et al. 2013). Cho et al. confirmed that FN III-1c region can activate integrin through PI3K-AKT signaling pathway α v β 5 to enhance cancer cell adhesion[Cho et al. 2016]. Our results indicated that ITGB5 functions through cell adhesion signal pathway, which was similar to other previous studies. Manipulation of the cell adhesion signal pathway with some small molecule drugs may be a potential direction to overcome TSCC metastasis. In our study, we examined ITGB5 in TSCC tissue sections and found that it was overexpressed in cancerous lesions, especially in patients with lymph node metastasis. We firstly revealed the possible role of ITGB5 by tracing its upstream regulatory factors and downstream regulatory pathways in promoting metastasis in TSCC.

In the near future, we may examine more TSCC tissues to further confirm critic role of ITGB5 in promoting metastasis. If possible, we may also perform some clinical trials to further excavate the role of ITGB5 in the TSCC patients. Assessment of ITGB5 in the future clinical practice may make early diagnosis of TSCC possible or even provide therapeutic targets to suppress TSCC metastasis.

Conclusion

ITGB5 was overexpressed in TSCC patients with lymph node metastasis, which indicated a worse prognosis. ROS enhanced the expression of ITGB5 to promote to metastasis of TSCC through EMT, thus scavenge of ROS or knock down of ITGB5 may be the strategies to overcome metastasis of TSCC. In view of the role of ITGB5 in adhesion signal pathway, screening for some small molecule drugs targeting ITGB5-relative pathway may be a potential direction to overcome TSCC metastasis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00432-024-05922-z.

Author contributions W.C. and T.J. conceptualized and designed the study. W.C. wrote the original manuscript. T.J. reviewed and edited the manuscript. W.C. and H.Y. carried out the experimentation. L.H. and F.L. performed formal analyses. C.F and L.Y. interpreted the data. All authors reviewed the manuscript.

Funding This work was supported by the National Natural Science Foundation of China (Grant No. 82002861 to WX.C.); the Guangdong Basic and Applied Basic Research Foundation (Grant No. 2021A1515010859 to TT.J.); the Guangdong Medical Science and Technology Research Fundation (Grant No. A2023115 to TT.J.); the Guangzhou Health Technology Foundation (20231A011007 to FX.L.); the Guangdong Basic and Applied Basic Research Foundation (Grant No. 2019A1515110124 to WX.C.).

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Sun Yat-sen Memorial Hospital (AP20220172) and Guangzhou First People Hospital (K-2022-092-02) with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Competing interests The authors declare no competing interests.

References

Al-Jamaei AAH, de Visscher JGAM, Forouzanfar T, Brakenhoff RH, Leemans CR, Stikvoort A, Zandieh-Doulabi B, Helder MN (2023) Radiation modulates expression and related activities of c-Met protein in oral tongue squamous cell carcinoma cell lines. J Cancer Res Clin Oncol 149(8):4173–4184

Bianchi A, Gervasi ME, Bakin A (2010) Role of beta5-integrin in epithelial-mesenchymal transition in response to TGF-beta. Cell Cycle 9(8):1647–1659

Bianchi-Smiraglia A, Paesante S, Bakin AV (2013) Integrin beta5 contributes to the tumorigenic potential of breast cancer cells through the Src-FAK and MEK-ERK signaling pathways. Oncogene 32(25):3049–3058

Chen JR, Zhao JT, Xie ZZ (2022) Integrin-mediated cancer progression as a specific target in clinical therapy. Biomed Pharmacother 155:113745

Cho C, Horzempa C, Jones D, McKeown-Longo PJ (2016) The fibronectin III-1 domain activates a PI3-Kinase/Akt signaling pathway leading to alphavbeta5 integrin activation and TRAIL resistance in human lung cancer cells. BMC Cancer 16:574

Choudhury SR, Byrum SD, Alkam D, Ashby C, Zhan F, Tackett AJ, Rhee FV et al (2023) Expression of integrin β-7 is epigenetically enhanced in multiple myeloma subgroups with high-risk cytogenetics. Clin Epigenetics 15(1):18

Cooper J, Giancotti FG (2019) Integrin signaling in Cancer: mechanotransduction, stemness, epithelial plasticity, and Therapeutic Resistance. Cancer Cell 35(3):347–367

Denadai MV, Viana LS, Affonso RJ, Silva SR, Oliveira ID, Toledo SR, Matos D (2013) Expression of integrin genes and proteins in progression and dissemination of colorectal adenocarcinoma. Bmc Clin Pathol 13:16

Fan S, Tian T, Chen W, Lv X, Lei X, Zhang H, Sun S, Cai L, Pan G, He L, Ou Z, Lin X, Wang X, Perez MF, Tu Z, Ferrone S, Tannous BA, Li J (2019) Mitochondrial miRNA determines Chemoresistance by Reprogramming metabolism and regulating mitochondrial transcription. Cancer Res 79(6):1069–1084

Fang Q, Li P, Qi J, Luo R, Chen D, Zhang X (2019) Value of lingual lymph node metastasis in patients with squamous cell carcinoma of the tongue. Laryngoscope 129(11):2527–2530

Gu WC, Sun H, Zhang M, Mo SC, Tan C, Ni SJ, Yang ZC, Wang YL, Sheng WQ Wang L(2023) ITGB1 as a prognostic biomarker correlated with immune suppression in gastric cancer. Cancer Med 12(2):1520–1531

Hirano T, Shinsato Y, Tanabe K, Higa N, Kamil M, Kawahara K, Yamamoto M, Minami K, Shimokawa M, Arigami T, Yanagita S, Matushita D, Uenosono Y, Ishigami S, Kijima Y, Maemura K, Kitazono I, Tanimoto A, Furukawa T, Natsugoe S (2020) FARP1 boosts CDC42 activity from integrin alphavbeta5 signaling and correlates with poor prognosis of advanced gastric cancer. Oncogenesis 9(2):13

Huang WC, Jang TH, Tung SL, Yen TC, Chan SH, Wang LH (2019) A novel miR-365-3p/EHF/keratin 16 axis promotes oral squamous cell carcinoma metastasis, cancer stemness and drug resistance via enhancing beta5-integrin/c-met signaling pathway. J Exp Clin Cancer Res 38(1):89

Kurokawa A, Nagata M, Kitamura N, Noman AA, Ohnishi M, Ohyama T, Kobayashi T, Shingaki S, Takagi R (2008) Diagnostic value of integrin alpha3, beta4, and beta5 gene expression levels for the clinical outcome of tongue squamous cell carcinoma. Cancer 112(6):1272–1281

Lambert AW, Pattabiraman DR, Weinberg RA (2017) Emerging Biological principles of Metastasis. Cell 168(4):670–691

Lin Z, Sun L, Xie S, Zhang S, Fan S, Li Q, Chen W, Pan G, Wang W, Weng B, Zhang Z, Liu B, Li J (2019) Chemotherapy-Induced Long non-coding RNA 1 promotes metastasis and chemo-resistance of TSCC via the Wnt/beta-Catenin signaling pathway. Mol Ther 26(6):1494–1508

Liu PF, Chen HC, Shu CW, Sie HC, Lee CH, Liou HH, Cheng JT, Tsai KW, Ger LP (2020) Guanylate-binding protein 6 is a novel biomarker for tumorigenesis and prognosis in tongue squamous cell carcinoma. Clin Oral Investig 24(8):2673–2682

Lu Q, Wang WW, Zhang MZ, Ma ZX, Qiu XR, Shen M, Yin XX (2019) ROS induces epithelial-mesenchymal transition via the TGF-beta1/PI3K/Akt/mTOR pathway in diabetic nephropathy. Exp Ther Med 17(1):835–846

Lv Z, Yang Y, Yang C (2020) Integrin alpha7 correlates with worse clinical features and prognosis, and its knockdown inhibits cell proliferation and stemness in tongue squamous cell carcinoma. Int J Oncol 56(1):69–84

Pastushenko I, Blanpain C (2019) EMT Transition States during Tumor Progression and Metastasis. Trends Cell Biol

Setti A, Sankati HS, Devi TA, Sekhar AC, Rao JV, Pawar SC (2013) Structural insights into the extra cellular segment of integrinbeta5 and molecular interaction studies. J Recept Signal Transduct Res 33(5):319–324

Shen J, Xu J, Chen B, Ma D, Chen Z, Li J-C et al (2019) Elevated Integrinα6 expression is involved in the occurrence and development of lung adenocarcinoma, and predicts a poor prognosis: a study based onImmunohistochemical analysis and Bioinformatics. J Cancer Res Clin Oncol 145(7):1681–1693

Shi W, He J, Huang Y, Zeng Z, Feng Z, Xu H, Nie Y (2021) Integrin beta5 enhances the malignancy of human colorectal cancer by increasing the TGF-beta signaling. Anticancer Drugs 32(7):717–726

Slack RJ, Macdonald S, Roper JA, Jenkins RG, Hatley R (2022) Emerging therapeutic opportunities for integrin inhibitors. Nat Rev Drug Discov 21(1):60–78

Stojanovic N, Brozovic A, Majhen D, Bosnar MH, Fritz G, Osmak M, Ristov AA (2016) Integrin alphavbeta3 expression in tongue squamous carcinoma cells Cal27 confers anticancer drug resistance through loss of pSrc(Y418). Biochim Biophys Acta 1863(8):1969–1978

Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z (2017) GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 45(W1):W98–102

Wortzel I, Dror S, Kenific CM, Lyden D (2019) Exosome-mediated metastasis: communication from a Distance. Dev Cell 49(3):347–360

Zhang LY, Guo Q, Guan GF, Cheng W, Cheng P, Wu AH (2019) Integrin Beta 5 is a prognostic biomarker and potential therapeutic target in Glioblastoma. Front Oncol 9:904

Zhang YJ, Dho SE, Othman K, Simpson CD, Lapierre J, Bondoc A, McGlade CJ (2022) Numb exon 9 inclusion regulates Integrinβ5 surface expression and promotes breast cancer metastasis. Oncogene 41(14):2079–2094

Zhuge L, Cai H, Huang Z, Wang S, Li Z (2023) The optimal number of examined lymph nodes for accurate nodal staging and favorable prognosis of oral tongue squamous cell carcinoma. Oral Oncol 140:106368