

Knockdown of Gab1 Inhibits Cellular Proliferation, Migration, and Invasion in Human Oral Squamous Carcinoma Cells

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Grb2-associated binder 1 (Gab1) is often aberrant in cancerous cells and tissues, whose alteration is responsible for aggressive phenotypes. In this study, we examined the Gab1 expression in human oral squamous cell carcinoma (OSCC) tissues and investigated the cellular and molecular effect of Gab1 on migration, invasion, and cell growth of the OSCC cell lines SCC15 and SCC25. We found that Gab1 was overexpressed in OSCC tissues and cells, which is related to the protein levels of various molecules associated with cellular proliferation, migration, and invasion. Functional assays identified that Gab1 overexpression promoted cell proliferation and invasion of OSCC cells and inhibited cell apoptosis in the SCC15 and SCC25 cell lines. On the other hand, Gab1 silencing affected the proliferation and invasion of OSCC cells and induced cell apoptosis. Western blot assay identified that Gab1 overexpression suppressed the expression of Cdc20 homolog 1 (Cdh1) and then promoted cell invasion in OSCC cells. Furthermore, Gab1-mediated Cdh1 downregulation was significantly reversed when the cells were subjected to an inhibitor of p-Akt. In conclusion, these results suggested that Gab1 induced malignant progression of OSCC cells probably via activation of the Akt/Cdh1 signaling pathway. Thus, Gab1 may be a potential therapeutic target in the treatment of OSCC patients.

Key words: Grb2-associated binder 1 (Gab1); Cdc20 homolog 1 (Cdh1); Malignance; Oral squamous cell carcinoma (OSCC)

INTRODUCTION

Oral squamous cell carcinoma (OSCC) leads to a portion of head and neck carcinomas, which may present as a primary lesion in any portion of the oropharynx or oral cavity. The results from the International Agency for Research on Cancer demonstrated that the number of new cases of OSCC and correlated deaths was 300,373 and 145,353, respectively¹. OSCC has been reported as a major cause of mortality and morbidity in patients with neck and head cancer². Despite the great improvement in detection, localized diagnosis, and disease treatments witnessed over the past decades, the overall survival rate of OSCC has merely increased by 5%^{3,4}, and the long-term survival rate of OSCC patients is still unfavorable. It should be noted that growth factor receptor bound protein 2 (Grb2)-associated binder 1 (Gab1) correlated with cancer development^{5,6}. However, the functions and mechanism of Gab1 in OSCC cell growth and metastasis are still unclear.

Structure–function studies have revealed key roles for Grb2, Src-homolog 2 domain-containing Shc, and Gab1 adaptor proteins downstream of the Met receptor

in various biological events, which, if not strictly coordinated, may contribute to the development and progression of cancers⁷. Recruitment of the Grb2 or Shc adaptor proteins is required and sufficient for oncogenic transformation, anchorage-independent growth, and experimental metastasis by the Met receptor oncoprotein⁸. Shc also plays a critical role in promoting the production of vascular endothelial growth factor and the early onset of tumor angiogenesis by the Met receptor⁹. Alternatively, the recruitment of the docking protein Gab1 is essential for Met receptor-mediated invasive branching morphogenesis of epithelial cells. In cancer cells, upregulation of Gab1 in cancer cells can induce the activation of many key pathways involving the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways, which are implicated in cell proliferation, apoptosis, migration, and invasion^{10,11}.

In this work, we identified that Gab1 protein was highly expressed in OSCC tissues. At the same time, we demonstrated that upregulation of Gab1 promoted cell proliferation, migration, and invasion and inhibited cell apoptosis of SCC15 and SCC25 cells. These results indicated that

Gab1 may be a potential therapeutic target in the treatment of OSCC patients.

MATERIALS AND METHODS

Ethics Statement

The study was conducted with permission from the medical ethics committee of Rizhao People's Hospital, Jining Medical University. Written, informed consent was acquired from all subjects or guardians prior to using their resected specimens.

OSCC Tissues

A total of 30 pairs of resected OSCC and corresponding normal oral epithelial tissues were collected from patients who were diagnosed in the Department of Stomatology, Rizhao People's Hospital, Jining Medical University (Rizhao, P.R. China) from 2010 to 2016. Ethical approval was obtained from the hospital, as well as fully informed consent from all patients prior to sample collection. None of the patients had received chemotherapy or radiotherapy before surgery. All tissue samples were confirmed by pathological examinations. Fresh frozen tissues were stored in liquid nitrogen until use. Clinical parameters, including pathological features and metastasis, were retrospectively collected by reviewing patients' charts. This study was approved by the Review Board of the Hospital Ethics Committee.

Cell Cultures

Primary normal human oral keratinocyte (NHOK) cells were cultivated as previously described.¹² Human OSCC cell lines SCC15, SCC25, HN4, and HN6 (American Type Culture Collection, Manassas, VA, USA) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml) (Sigma-Aldrich, Poole, UK). Cells were incubated at 37°C with 95% humidity in 5% CO₂.

Cell Transfection

To generate Gab1 knockdown stable cell lines, Gab1 short interfering RNAs (siRNAs) were synthesized and purchased from Invitrogen. Twenty-four hours prior to transfection, cells were plated onto a six-well plate at 70%–90% confluency. Cells were then transfected with siRNA at a final concentration of 50 nM with the Dharma FECT reagent (Thermo Fisher Scientific, Lafayette, CO, USA) according to the manufacturer's instructions. Cells were also incubated with dimethyl sulfoxide (DMSO; vehicle) or the Akt inhibitor LY294002 and si-cell division cycle 20 (Cdc20) homolog 1 (si-Cdh1) to explore possible pathways through which Gab1 may act. After 6 h of incubation at 37°C, the transfection medium was

replaced with 2 ml of complete medium containing 10% FBS. Cells were collected for the following experiments at the indicated times.

Western Blot Analysis

Total protein from tissue and cells was harvested in radioimmunoprecipitation assay (RIPA) lysis buffer. After centrifugation at 12,000 rpm for 10 min, the protein concentration was measured by the BCA Protein Assay Kit (Pierce, Appleton, WI, USA). Then 30 µg of total protein was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA) and incubated with blocking buffer for 60 min at room temperature and then incubated with primary antibody at 4°C overnight. Antibodies for the following proteins were used: Gab1, β-actin (as loading control), Ki-67, Bcl-2 (B-cell lymphoma 2), cyclin D1, Bcl-2-associated X protein (Bax), tissue inhibitor of matrix metalloproteinases 2 (TIMP2), Cdh1, phosphorylated protein kinase B (p-Akt), and total Akt (t-Akt). Finally, proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibody. Western blot quantitative analysis was performed by Scion Image software (Scion Corporation, Frederick, MD, USA).

RNA Extraction and Qualitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from tissue and cells using Tripure Isolation Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA using the Transcriptor First-Strand cDNA Synthesis Kit (Roche). RT-PCR was performed to detect Gab1 expression in triplicates with SYBR Green I Master (Roche). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Cell Proliferation Assay

Cells were seeded at 2,000 per well in 96-well plates and cultured after transfection. Cell proliferation was detected at the indicated time points using a cell counting kit-8 (CCK-8; Dojindo Laboratories, Rockville, MD, USA) following the manufacturer's instructions. All assays were performed in octuplicate and repeated at least three times.

Cell Apoptosis Assay

Cells were treated as mentioned above. After 36 h, cells were harvested, washed, and resuspended with binding buffer (Sungene-BASF, Ludwigshafen, Germany). Cells were incubated with annexin V-fluorescein isothiocyanate (FITC) for 20 min and propidium iodide (PI) for

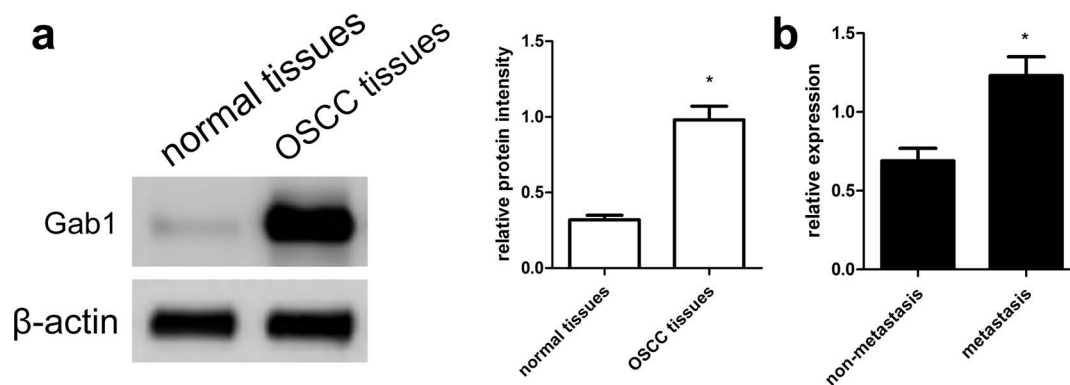


Figure 1. The expression of growth factor receptor bound protein 2 (Grb2)-associated binder 1 (Gab1) in oral squamous cell carcinoma (OSCC) tissues. (a) Western blot analysis was used to assess the protein levels of Gab1 in 30 pairs of OSCC tissues and adjacent normal oral epithelial tissues. (b) The expression of Gab1 in metastasis and nonmetastasis OSCC tissues was assessed. Relative Gab1 expression was normalized to β -actin. Results represent mean \pm SEM. * $p < 0.001$ compared to normal tissues or nonmetastasis. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test.

5 min at room temperature in darkness before analyzing with a flow cytometer.

Migration and Invasion Assays

For the migration assay experiments, we seeded cells on the upper chamber of each insert in 500 μ l of DMEM, and then 500 μ l of DMEM and 10% FBS was added to a 24-well plate for 12 h of incubation at 37°C. The cells on the lower layer were collected and fixed with 0.1% crystal violet. For the invasion assay, Transwell chambers were uniformly plated with 60 μ l of Matrigel diluted with DMEM and then incubated for 4 h at 37°C, and then the same procedures with migration assay were conducted.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Each experiment was performed at least three times. The data were expressed as mean \pm SD. One-way ANOVA followed by the Dunnett's multiple comparisons test for between-group analysis was used where there were more than two groups tested, while an unpaired Student's *t*-test was used to determine the significant differences of all remaining results using SPSS v17. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Gab1 Is Highly Expressed in Human OSCC Tissues

To explore the relationship between Gab1 expression and OSCC, the expression of Gab1 was detected in 30 pairs of OSCC tissues and adjacent normal oral epithelial tissues using Western blot. As shown in Figure 1a, the expression level of Gab1 was significantly increased in OSCC tissues compared with normal oral epithelial tissues ($p < 0.001$). Also, the expression of Gab1 was found

to be obviously increased in OSCC tissues with lymph node metastasis ($n = 12$) compared with tissues without lymph node metastasis ($n = 18$; $p < 0.001$) (Table 1, Fig. 1b). However, the expression of Gab1 was not associated with gender or tumor location ($p > 0.05$) (Table 1). These data indicated that Gab1 may play a role in the development of OSCC.

Gab1 Is Highly Expressed in Human OSCC Cells

To determine the role of Gab1 in human OSCC, we first analyzed the expression of Gab1 using qRT-PCR and Western blot for human nontumor keratinocyte NHOK and OSCC cell lines (SCC15, SCC25, HN4, and HN6). Compared with NHOK cells, Gab1 was expressed at higher mRNA levels in all of the OSCC cell lines tested (Fig. 2a). Consistently, compared with NHOK cells, Gab1

Table 1. Association Between Grb2-Associated Binder 1 (Gab1) and Pathological Characteristics of Oral Squamous Cell Carcinoma (OSCC) Patients

Pathological Indicators	<i>n</i>	Gab1 Expression	<i>p</i> Value
Age (years)			
>50	11	1.02 (0.11)	0.556
≤ 50	19	0.89 (0.09)	
Gender			
Male	15	0.99 (0.11)	0.896
Female	15	0.97 (0.09)	
Lymph nodes metastasis			
Yes	12	1.23 (0.12)	0.001
No	18	0.76 (0.08)	
Location			
Epithelium	5	1.09 (0.11)	0.058
Epithelial and mesenchymal	25	0.97 (0.09)	

Statistical significance was assessed using an unpaired two-tailed Student's *t*-test.

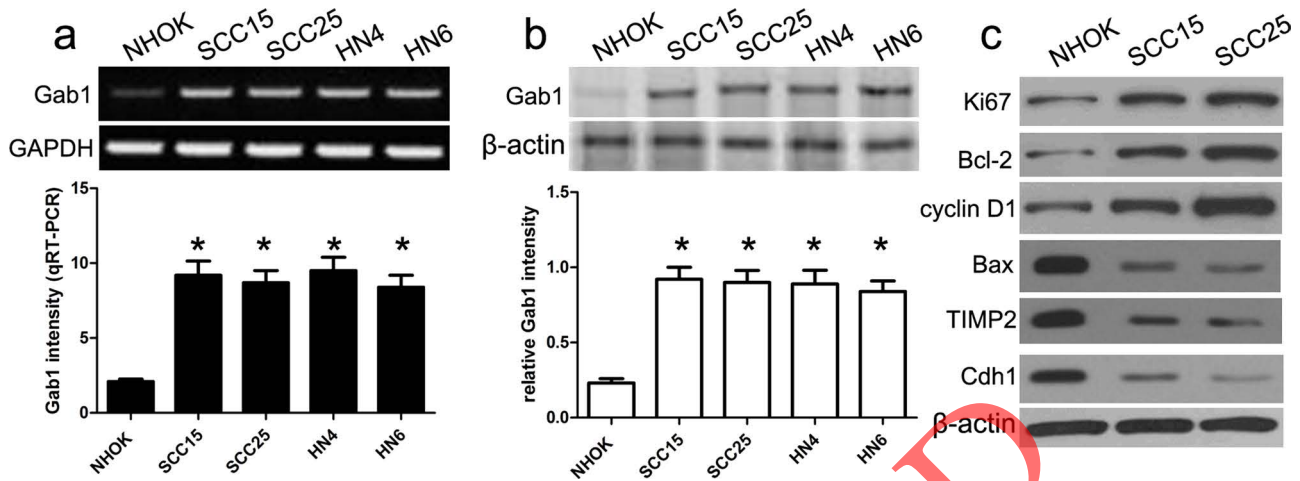


Figure 2. The expression of Gab1 in OSCC cells and the expression of malignant progression-related protein markers. (a) Determination of endogenous expression of Gab1 mRNA by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) in OSCC cells lines SCC15, SCC25, HN4, and HN6, and the human nontumor cell line NHOK (normal human oral keratinocytes). (b) Western blot analysis was used to assess the protein levels of Gab1 in OSCC cells lines SCC15, SCC25, HN4, and HN6, and the human nontumor cell line NHOK. (c) Western blot analysis was used to assess the protein levels of Ki-67, B cell lymphoma 2 (Bcl-2), cyclin D1, Bcl-2-associated X protein (Bax), tissue inhibitor of matrix metalloproteinases 2 (TIMP2), and Cdc20 homolog 1 (Cdh1) in SiHa, SCC25, and the human nontumor line NHOK. * $p < 0.001$ compared to NHOK cells. Statistical significance was assessed using one-way ANOVA followed by the Dunnett's multiple comparisons test for between-group analysis.

was expressed at higher protein level in all the OSCC cell lines tested (Fig. 2b). To further determine the roles of Gab1, SCC15 and SCC25 cell lines were selected as a model, in which the expression of Gab1 was more stable than the other two OSCC cell lines.

Furthermore, we quantified the protein levels of various molecules associated with cellular proliferation, apoptosis, migration, invasion, and survival in SCC15 and SCC25 cell lines with expression of Gab1. SCC15 or SCC25 cells exhibited decreased protein levels of TIMP2, Bax, and Cdh1 when compared with those of NHOK cells. On the other hand, SCC15 and SCC25 cells exhibited increased protein levels of Ki-67, Bcl-2, and cyclin D1 when compared with those of NHOK cells (Fig. 2c). These data indicated that Gab1 plays a role in the malignant progression of human OSCC.

Gab1 Promotes the Proliferation of SCC15 and SCC25 Cells

To explore whether Gab1 contributes to the proliferation of OSCC cells in vitro, we detected cell proliferation levels using SCC15 and SCC25 cells with knockdown of Gab1. Specific-stranded RNA oligonucleotides against Gab1 or negative RNA control were transfected into SCC15 and SCC25 cells, and then a rapid downregulation of Gab1 mRNA and protein was observed with the specific RNA against Gab1. Cell proliferation assays showed a statistically significant decrease in proliferation

when Gab1 was downregulated in SCC15 and SCC25 cells compared with si-control (Fig. 3a).

Using the apoptosis assay, we found that the apoptosis proportions in SCC15 and SCC25 cells with the treatment of si-Gab1 were significantly increased (Fig. 3b). Western blot analysis verified that the knockdown of Gab1 increased the expression level of the proapoptotic protein Bax and decreased the expression level of the antiapoptotic protein Bcl-2 in SCC15 and SCC25 cells (Fig. 3c).

Gab1 Promotes Cell Migration and Invasion of SCC15 and SCC25 Cells

Transwell assays frequently were utilized to determine the migration and invasiveness of OSCC cells in vitro. We therefore analyzed the migration and invasiveness of OSCC cells in vitro using Transwell assays. In this study, the ability of SCC15 cells with si-Gab1 to migrate to the lower layer or invade into Matrigel-coated matrix significantly decreased compared with SCC15 cells with si-control (Fig. 4a and b). Similar results were obtained in SCC25 cells as shown in Figure 4.

Cdh1 Plays an Important Role in Gab1-Mediated Migration and Invasion

To further determine whether Gab1 could regulate Cdh1 expression in OSCC cells, si-Gab1 was transiently transfected into OSCC SCC15 cell lines. Western blot analysis was conducted to detect the expression of Gab1.

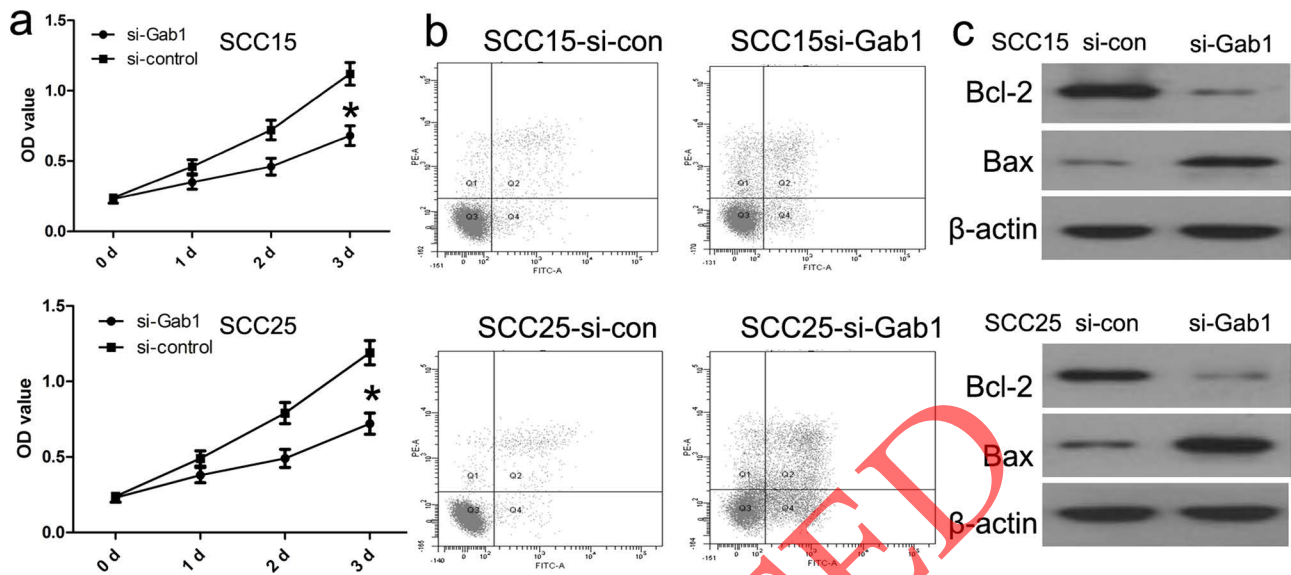


Figure 3. Gab1 expression promoted the proliferation of SCC15 and SCC25 cells. (a) Total cell number assays were performed using cell counting kit-8. Cells were seeded in full serum, and the total cell number was counted every 12 h. (b) Flow cytometry assays showed a statistically significant increase in apoptosis when Gab1 was knocked down in SCC15 and SCC25 cells. (c) Western blot analysis was used to assess the protein levels of Bcl-2 and Bax in SCC15 and SCC25 cells transfected with small interfering RNA for Gab1 (si-Gab1) or si-control. Results represent mean \pm SEM. * $p < 0.001$. Statistical significance was assessed using ($p < 0.05$ was considered as significant compared to si-control, using an unpaired two-tailed Student's *t*-test) SPSS 17.0.

The knockdown of Gab1 in SCC15 cell lines decreased the expression level of p-Akt but increased the expression of Cdh1 (Fig. 5a). These data indicated that Gab1 could regulate Cdh1 expression in OSCC cells. To further elucidate the Gab1/Akt/Cdh1 pathway, we blocked the Akt or Cdh1 using the Akt inhibitor LY294002 or si-Cdh1, respectively, and found that LY294002 inhibited the expression of p-Akt but increased the expression of Cdh1 and did not affect Gab1 (Fig. 5b); on the other hand, si-Cdh1 did not affect the expression of p-Akt and Gab1, indicating that there is a Gab1/Akt/Cdh1 pathway that regulates the tumor biology (Fig. 5c).

Subsequently, we analyzed whether Cdh1 mediated the effect of Gab1 on cell migration and invasion in SCC15 cells. We used the specific siRNA to knock down Gab1 or Cdh1 expression in SCC15 cells; at the same time, we used LY294002 to inhibit the expression of p-Akt. We performed cell migration and invasion assays and found that the migration and invasion abilities in SCC15 cells transfected with si-Gab1 were significantly decreased compared with that in SCC15 cells transfected with si-control (Fig. 6a). Moreover, the inhibition of the p-Akt pathway significantly attenuated the cell migration and invasion abilities in SCC15 cells (Fig. 6b). Importantly, the migration and invasion abilities in SCC15 cells with si-Cdh1 were significantly stronger than those in SCC15 cells with si-control (Fig. 6c). These results demonstrated that the expression of Gab1 could induce cell migration

and invasion via activation of the Akt/Cdh1 signaling pathway. Similar results also were obtained in SCC25 cells (data not shown).

DISCUSSION

In this study, we reported that the Gab1 protein was highly expressed in OSCC tissues and cells. Some studies identified that Gab1 expression strongly correlated with poor prognosis in various tumors, which indicated that Gab1 could be a potentially useful diagnostic biomarker or therapeutic target for OSCC. In this study, we demonstrated that Gab1 functionally promoted the malignant progression of OSCC cells. Low expression of Gab1 inhibited the proliferation and invasion and promoted apoptosis in SCC15 and SCC25 cells. Our results indicated that Gab1 stimulated an invasive phenotype in OSCC cells through Akt-mediated repression of Cdh1.

Recent studies have demonstrated that Gab1 plays an important role in cell proliferation and correlates with the invasion and metastasis of a wide variety of solid cancers such as lung, stomach, liver, and bladder tumors¹³. Increased Gab1 protein levels have been associated with advanced-stage human cancers or enhanced proliferation^{14,15}.

Gab1 amplification was previously observed in non-small cell lung and gastric cancers, strongly suggesting that Gab1 is an oncogene in multiple cancers, and the data from previous articles show that Gab1 expression

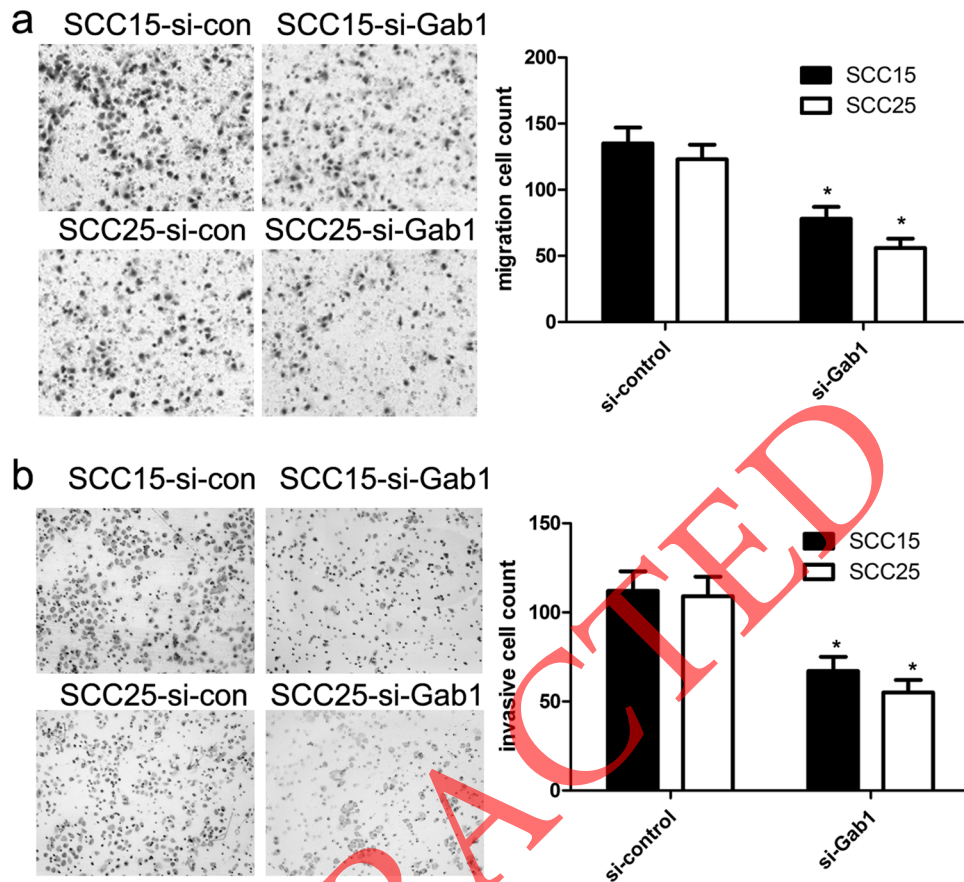


Figure 4. Gab1 regulates cell migration and invasion of OSCC cells. Transwell migration (a) and invasion (b) assays were carried out in SCC15 and SCC25 cells with depleted expression of Gab1 (si-Gab1). Each bar represents the mean \pm SEM of three independent experiments. * $p < 0.001$, compared with si-control, using an unpaired two-tailed Student's *t*-test.

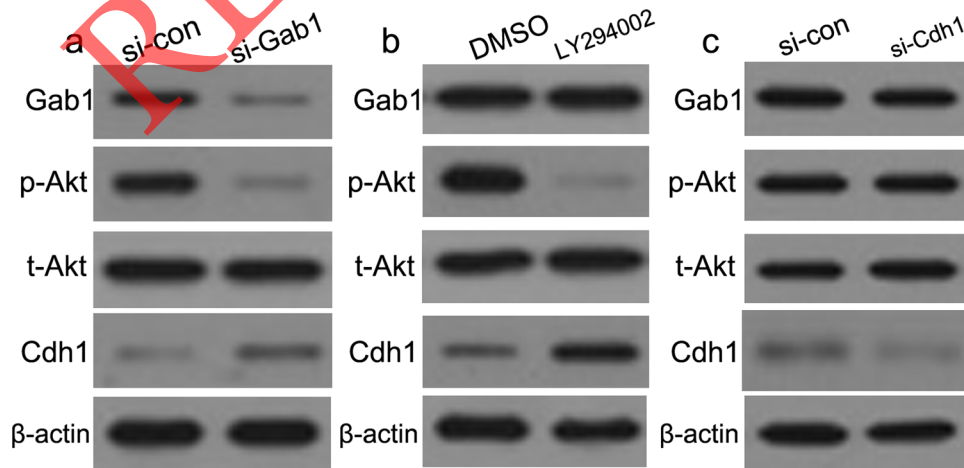


Figure 5. Gab1 induces protein kinase B (Akt)/cell division cycle 20 (Cdc20) homolog 1 (Cdh1) pathways in SCC15 cells. (a) Western blot analysis was used to assess the levels of Gab1, Cdh1, phosphorylated (p)-Akt, and total (t)-Akt in OSCC SCC15 cells with depleted expression of Gab1. (b) Western blot analysis was used to assess the levels of Gab1, Cdh1, p-Akt, and Akt in OSCC SCC15 cells in the presence of Akt activity inhibitor LY294002. (c) Western blot analysis was used to assess the levels of Gab1, Cdh1, p-Akt, and Akt in OSCC SCC15 cells with depleted expression of Cdh1.

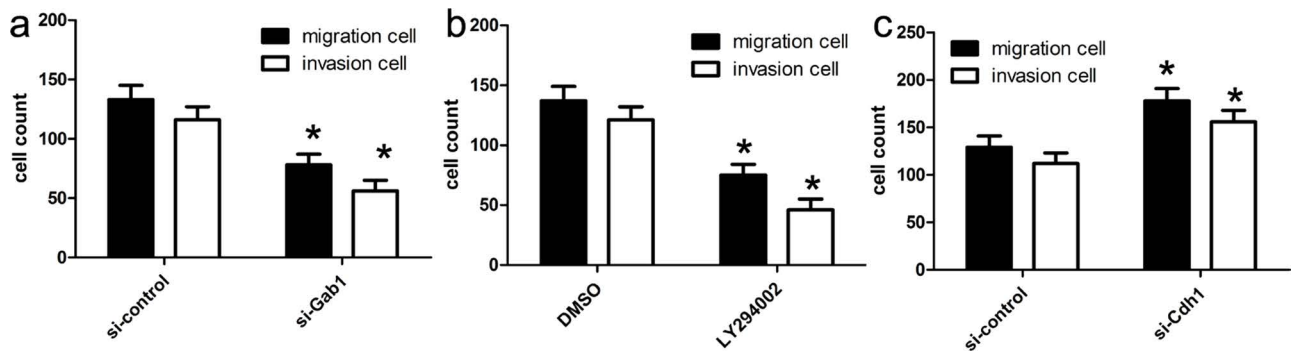


Figure 6. Gab1/Akt/Cdh1 regulates cell migration and invasion of OSCC SCC15 cells. (a–c) Transwell migration and invasion assays were carried out in SCC15 and SCC25 cells with different treatments as mentioned in Figure 4. Each bar represents the mean \pm SEM of three independent experiments. * $p < 0.001$, compared with si-control or dimethyl sulfoxide (DMSO), using an unpaired two-tailed Student's *t*-test.

correlates with poor prognosis^{14,15}. Gab1 promotes cancer cell proliferation, survival, oncogenicity, and invasion in various cancers, such as mammary carcinoma, gastric cancer, and liver cancer^{16–18}.

For the first time, we found that high expression of Gab1 decreased protein expression of Bax, TIMP2, and Cdh1, but increased protein levels of Ki-67 and Bcl-2, which closely correlated with increasing cell cycle progression, antiapoptosis, proliferation, metastasis, and invasion of OSCC cells. siRNA-mediated depletion of Gab1 induced the apoptosis of OSCC cells by decreasing the antiapoptotic protein Bcl-2 and increasing the proapoptotic protein, Bax. The ratio of the antiapoptotic and proapoptotic members within the Bcl-2 family plays an important role in cell survival.

It is well known that Gab1 is associated with invasion and metastasis, which play very important roles in the progression of tumors. Using Transwell analysis, we found that Gab1 exerts a critical effect on the migration and invasion of OSCCs. Moreover, we observed that Gab1 overexpression repressed Cdh1 expression to promote cell migration and invasion in OSCC cells. Western blot analysis showed that siRNA against Gab1 increased the expression of Cdh1, and decreased phosphorylation of Akt can upregulate the expression of the Cdh1 protein. Furthermore, Cdh1 was significantly upregulated by virtue of an Akt inhibitor. Together with previous reports, our results suggested that Gab1 induced the migration and invasion of OSCC cells probably by inducing the Akt/Cdh1 signaling pathway^{19–21}. Cdh1 is a downstream protein of Gab1 and may be a key modulator of Gab1-mediated OSCC invasion.

In conclusion, these results suggested that Gab1 induced malignant progression of OSCC cells probably via activation of the Akt/Cdh1 signaling pathway. Thus, Gab1 may be a potential therapeutic target in the treatment of OSCC patients.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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