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Functional characterization of GNA11 in esophageal squamous cell carcinoma: bioinformatics and cellular functional analysis

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ABSTRACT: Guanine nucleotide-binding protein subunit alpha 11 (GNA11) has an established role in various types of cancer, however, its involvement in esophageal squamous cell carcinoma (ESCC) remains uncharted. Data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) project were utilized to analyze GNA11 expression across various cancers, its functional enrichment pathways in ESCC, and its correlation with immune infiltration. Single-cell RNA sequencing was used to quantify GNA11 expression across different cell types. Real-time quantitative PCR and Western blotting were conducted to verify the GNA11 expression levels. ESCC cells KYSE150 and TE1 were transduced to construct knockdown and overexpression models. Proliferation was assessed using growth curve analysis, Cell Counting Kit-8 (CCK8) assays, and colony formation assays. Wound healing and transwell assays evaluated the impact of GNA11 on cancer cell migration and invasion. Functional enrichment analysis revealed associations with key pathways. Additionally, GNA11 interacts with proteins involved in G protein-coupled receptor and chemokine signaling pathways. Single-cell RNA sequencing and immune infiltration analyses indicated a positive correlation between GNA11 expression and tumor microenvironment components. *In vitro* experiments showed that GNA11 overexpression enhanced the proliferation, migration, and invasion capabilities of ESCC cells, whereas GNA11 knockdown produced the opposite effects. This study is the first to elucidate the critical role of GNA11 in the development and progression of ESCC. Abnormally high expression of GNA11 promotes ESCC and potentially influences interactions between stromal cells and malignant cells. Thus, GNA11 could serve as a promising therapeutic target for ESCC treatment.

KEYWORDS: esophageal squamous cell carcinoma, ESCC, GTP-binding protein alpha subunits, GNA11, cancer driver gene, tumor microenvironment

INTRODUCTION

Esophageal cancer (EC) is a prevalent malignancy of the digestive tract, ranking seventh in global incidence and sixth in mortality [1]. The majority of cancer cases, approximately 78%, are reported in Asia, with China alone contributing to around 49% of the total burden [2]. Within China, esophageal squamous cell carcinoma (ESCC) is the predominant subtype [3], and the symptoms of ESCC are often imperceptible in the early stages, leading to diagnoses typically being made in the middle or advanced stages for most patients [4]. Despite notable advancements in clinical treatment and targeted therapies, the prognosis for ESCC remains unsatisfactory. Therefore, it is crucial to delve into and elucidate the underlying mechanisms that drive the initiation and progression of ESCC.

As *GNA11* is identified as a cancer driver gene, the protein it encodes belongs to the guanine nucleotide-binding protein (G protein) family, which plays a regulatory or transduction role in various transmembrane signaling systems [5–7], and is closely associated with the progression of various cancers [8–10]. In previous studies, cancer driver gene-based protein microarray, multiple rounds of enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) assays showed that the anti-GNA11 IgG antibody was highly expressed in the serum of ESCC patients and could be used as a

diagnostic marker for ESCC, indirectly proving that the abnormal expression of GNA11 may occur during the occurrence of ESCC [11, 12]. Furthermore, immunohistochemical analysis of ESCC tissues and paired paracancerous tissues further confirmed the abnormally high expression of GNA11 protein in ESCC [12], suggesting a potential close association between GNA11 protein and the occurrence and progression of ESCC. While GNA11 frequently mutates in esophageal cancer and is linked to important cell signaling pathways such as PI3K, RAS, and MAPK pathways [13], its specific mechanism of action in histological subtypes of ESCC remains unverified. Therefore, the purpose of this study was to characterize the potential role of GNA11 in the development of ESCC through bioinformatics analysis and functional experiments conducted *in vitro*.

MATERIALS AND METHODS

Data acquisition

The RNA-seq data from the combined TCGA and GTEx cohorts for pan-cancer analysis were downloaded from the UCSC Xena database (https://xena.ucsc.edu/) [14]. A total of 8,254 cancer samples and 7,625 normal samples were included. Specifically, a total of 158 esophageal carcinoma (ESCA) tissue samples (including 80 ESCC tissue samples for a particular analysis) and 657 corresponding normal tissue samples were utilized for subsequent bioinformatics analysis.

Functional enrichment analysis

Using RNA-seq data from 80 ESCC samples, GNA11 high and low expression groups were defined based on median expression levels, and differentially expressed genes (DEGs) were identified using the "limma" R package. Enrichment analyses were conducted using gene ontology (GO) and gene set enrichment analysis (GSEA) with the "clusterProfiler" R package [15]. An adjusted p value < 0.05 was considered significant. The protein-protein interaction (PPI) network was constructed for GNA11 in humans using the STRING database (https://string-db.org/), setting to display the top 50 interacting proteins (interaction score>0.7). The hub genes were identified with the Maximal Clique Centrality (MCC) method in Cytoscape (version 3.9.1).

Single cell and immunoinfiltration analysis

Single-cell RNA sequencing (scRNA-seq) analysis was performed using the Tumor Immune Single-cell Hub 2 (TISCH2) database (http://tisch.comp-genomics.org/home/) [16]. EPIC, MCPcounter, and Xcell algorithms were used to calculate the different immune cell infiltration scores in TIMER2.0 (http://timer.cistrome.org/) [17]. The correlation between GNA11 and immune infiltration was assessed using Pearson correlation coefficient.

Cell culture

The ESCA cell line (Eca109), ESCC cell lines (TE1, KYSE140, KYSE150) and human normal esophageal epithelial cell line (HEEC) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Twenty STR sites and sex identification sites were amplified from all the cell lines through the 21 CELLID System, and an ABI3130x1 genetic analyzer was used to detect and analyze the PCR products. The cells are free of mycoplasma infection. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS, Lonsera, UY), 1% penicillin-streptomycin, at 37 °C and 5% CO_2.

Western blot

Total proteins from the ESCC and HEEC cells were quantified using a BCA protein quantitative kit (Beijing Ding Guo Chang Sheng Biotechnology Co., Ltd., Beijing, China). The anti-GNA11 (Abcam, Hangzhou, China) and anti-tubulin (Wuhan Cusabio, Wuhan, China) antibodies were diluted at 1:100 and 1:5000, respectively, and secondary antibody at 1:5000. Protein bands were visualized using chemiluminescence (C300–C600) with an Azure Biosystems instrument following the manufacturer's instructions.

Quantitative real-time PCR (qRT–PCR)

Total RNA was extracted from the ESCC and HEEC cells using TRIzol reagent (Leagene Biotechnology Co., Ltd., Beijing, China), followed by cDNA synthesis with a NovoScriptPlus All-in-one 2st Strand cDNA Synthesis Supermix kit (Clontech Laboratories, Beijing, China). qPCR was performed using a SYBR Green Master Mix kit (Shanghai Novo Protein Scientific, Inc., Shanghai, China), with tubulin as the internal reference gene. The average expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. All steps were conducted as per the manufacturer's instructions.

The primer sequences: GNA11 forward, 5′-CTCT GCCAAGTACTACCTGAC-3′ and reverse, 5′-GATGATG TTCTCCAGGTCGAAA-3′; tubulin forward, 5′-CGGAG TCAACGGATTTGGTCGTAT-3′ and reverse, 5′-AGCCTT CTCCATGGTGGTGAAGAC-3′.

Establishment of stable ESCC cell lines with GNA11 knockdown and overexpression

The hU6-MCS-CBh-gcGFP-lentivirus containing target plasmids and the empty lentiviral control vector were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). The short hairpin (sh)RNA GNA11 sequence and control sequence were as follows: 5′-CGACAGCGACAAGATCATCTA-3′, 5′-TTCTCCGAAC GTGTCACGT-3′. The full-length human GNA11 sequence was used for overexpression. Transfection of the KYSE150 and TE1 cell lines was performed according to the manufacturer's instructions.

Cell proliferation assay

In the CCK-8 assay, KYSE150 and TE1 cells were respectively seeded into the 96-well plates at a concentration of 1×10^3 and 3×10^3 per well. When ESCC cells begin to adhere to the wall, the time is set to 0 h. At 0 h, 24 h, 48 h, 72 h, and 96 h, the prepared RPMI 1640 medium containing CCK-8 reagent was added to the 96-well plates, followed by incubation for another 2 h. The absorbance was measured at 450 nm. In the colony formation assay, cells were cultured in 6-well plates for 14 days at a density of 1000 cells per well until each single cell clone was between 0.3–1.0 mm and contained more than 50 cells. The cells were then stained with 0.5% crystal violet diluted with methanol for clone formation rate calculation. All steps were performed according to the manufacturer's instructions.

Cell migration and invasion assays

For the wound healing assay, KYSE150 and TE1 cells were seeded in 24-well plates and scratched when cells reached over 95% confluence (0 h). Images were taken at 0 h, 12 h, and 24 h and wound healing rates were calculated using Image J software. In the transwell assay, 100 µl of 6×10^4 cells were seeded into the upper chamber, and 600 µl RPMI-1640 medium containing 20% FBS was added to the bottom chamber. In the migration assay, 100 µl Matrigel matrix (diluted with RPMI 1640 basic medium at a ratio of 1:9) was added to the upper chamber. After 48 h of incubation, cells that migrated to the bottom of the filter were stained with crystal violet and counted in five randomly selected areas. All steps were performed according to the manufacturer's instructions.

Statistical analysis

R (version 4.2.2), GraphPad Prism 8.0 and IBM SPSS statistical software 25.0 were used in the study. Student's *t*-test or the Mann-Whitney U test was applied based on data distribution. Two-sided tests were conducted, and significance was set at $p < 0.05$.

RESULTS

The expression of GNA11 in pancancerous tissues and ESCC cells

In our analysis of *GNA11* gene expression across pancarcinoma tissues from the TCGA and GTEx cohorts, as outlined in the methods, we observed abnormal expression patterns in various tumors, with ESCA exhibiting significant overexpression ($p < 0.001$; Fig. 1). We further investigated the mRNA and protein expression levels of GNA11 in HEEC and four ESCA cell lines (Eca109, TE1, KYSE140, KYSE150) by qRT–PCR and Western blotting, where TE1 and KYSE150 are ESCC cell lines. As shown in Fig. 1b–d, except TE1, the mRNA and protein expression levels of GNA11 in ESCA and ESCC cells were greater than those in HEEC cells ($p < 0.05$).

Potential mechanisms of GNA11 in regulating the progression of ESCC

Following the screening criteria, 1133 upregulated DEGs and 40 downregulated DEGs with significant differences between the high and low GNA11 expression groups were identified ($p < 0.05$; Fig. 2a).

GO analysis revealed that the upregulated DEGs were significantly involved in extracellular matrix and structure organization, focal adhesion, integrin-mediated, Wnt and Ras protein signaling pathway, etc., while the downregulated DEGs were significantly involved in water homeostasis, keratinocyte and epidermal cell differentiation, etc. ($p < 0.05$; Fig. 2b,c). GSEA analysis (KEGG pathways) revealed that the ECM-receptor interaction, focal adhesion, PI3K-Akt signaling pathway and various cancer-related pathways comprised more upregulated DEGs than downregulated DEGs, significantly ($p < 0.05$; Fig. 2d). As shown in Fig. 2e, the PPI network of GNA11 showed that all related proteins were related to signal transduction, and most of them came from G protein-coupled receptor signaling pathways. The top hub genes (*GNAQ*, *GNB1*, *GNB3*, *GNG2*, *GNB2*, *GNB4*, *GNB5*, *PLCB2*, *CXCR4*) were enriched in pathways related to cancer. Among them, *GNB1*, *GNB3*, *GNG2*, *GNB2*, *GNB4* and *GNB5* were also related to the PI3K-Akt and Ras signaling pathway.

Single-cell expression and immunoinfiltration analysis of GNA11 in ESCC

In the ESCC dataset ESCA_GSE160269, the gene expression of GNA11 was predominantly observed in endothelial cells, followed by pericytes, fibroblasts, and malignant cells (Fig. 3a–c). Then, we used the EPIC, MCPcounter and Xcell algorithms to calculate the enrichment score for immune infiltration, and the results assessing their correlations with GNA11 are shown in Fig. 3d. Immunoinfiltration analysis revealed that cancer-related fibrocytes (CAFs), endothelial cells, the stroma score, and the microenvironment score were positively correlated with GNA11 expression. (Fig. 3e–h).

Changes in GNA11 expression affect the proliferation ability of ESCC cells

We successfully constructed GNA11 knockdown and overexpression cell models to further explore the biological function of GNA11 in ESCC (Fig. S1). As shown in Fig. 4a,b, the proliferation curves of the GNA11 knockdown groups demonstrated significantly lower proliferation rates compared to the control group, as assessed by the CCK-8 assay ($p < 0.05$). Conversely, the proliferation curve of the GNA11 overexpression group showed a significant increase compared to the control group ($p < 0.05$). Results from the colony

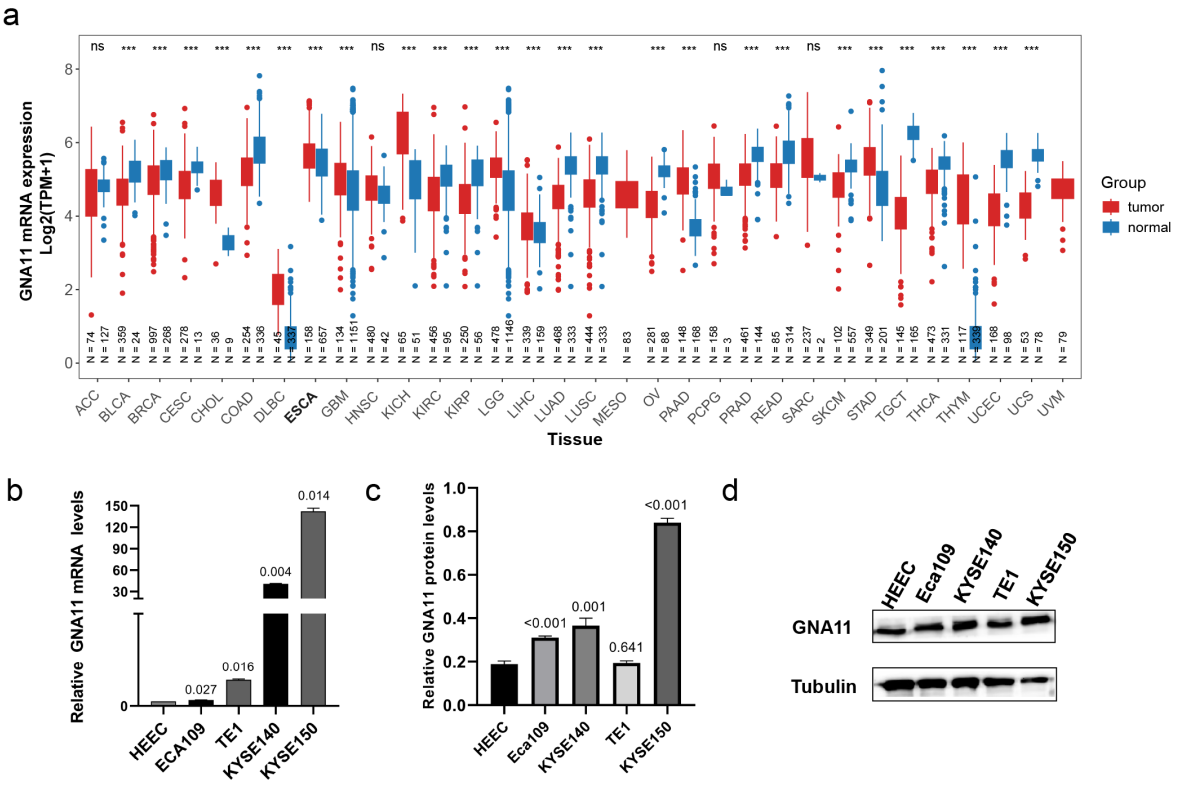


Fig. 1 The mRNA and protein expression of GNA11 in cancer tissues. (a) The mRNA expression of GNA11 in pan-cancerous tissues from the combined TCGA and GTEx cohorts. (b) The results of qRT-PCR and (c, d) Western blotting showed that the mRNA and protein expression of GNA11 increased in ESCA cells (Eca109, KYSE140, TE1, KYSE150) compared with that in normal esophageal epithelial cell lines (HEEC).

formation assay indicated a significant decrease in colony numbers with GNA11 knockdown and a significant increase with GNA11 overexpression ($p < 0.05$; Fig. 4c,d). These findings suggest that elevated GNA11 expression can enhance the proliferation ability of ESCC cells, while reduced expression has an inhibitory effect.

Changes in GNA11 expression affect the migration ability of ESCC cells

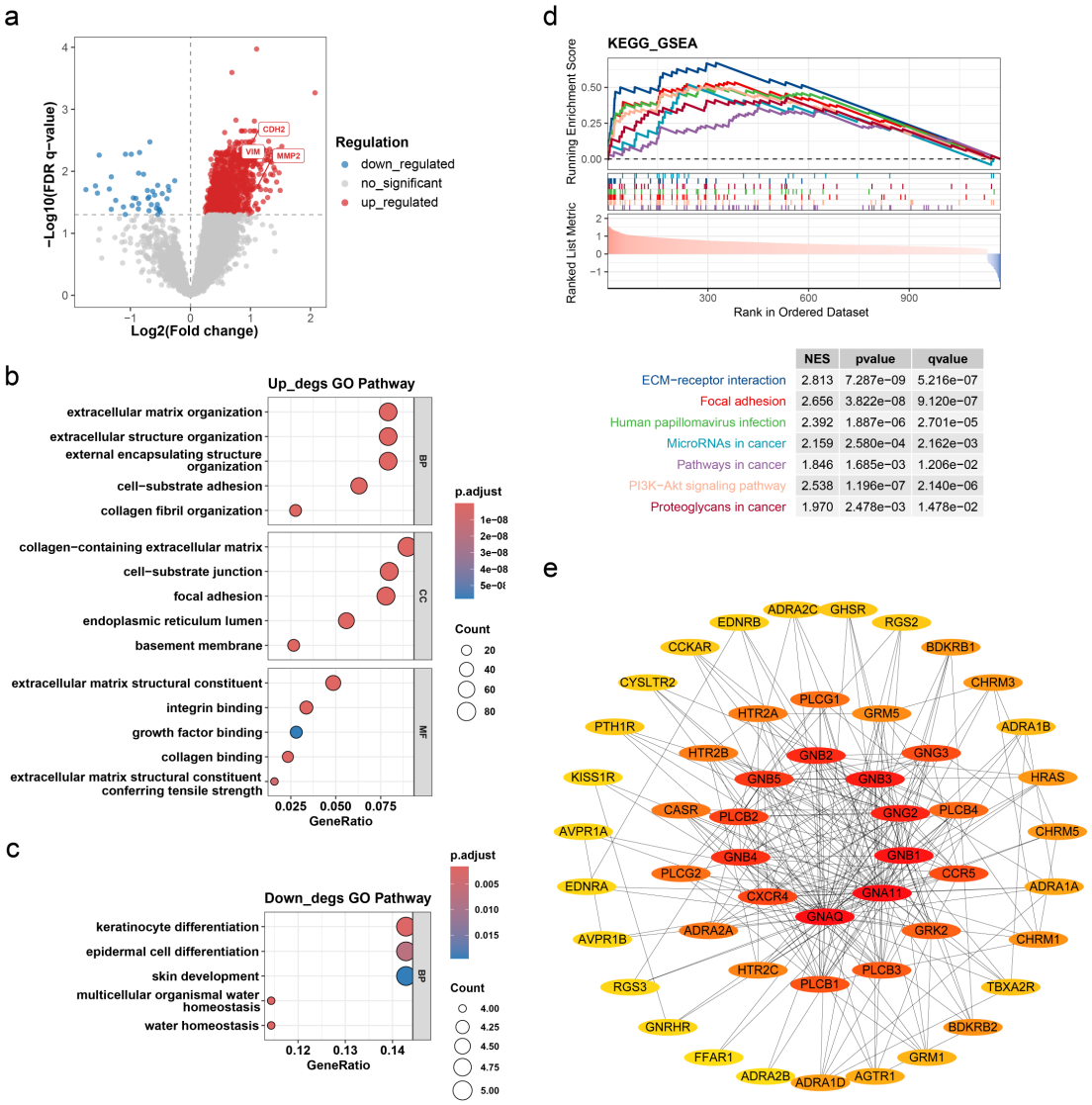
As shown in Fig. 5a,b, the wound healing assay and transwell migration assay revealed that GNA11 knockdown groups exhibited a lower wound healing rate compared to control cells, while GNA11 overexpression significantly enhanced the migration ability of ESCC cells ($p < 0.01$). Consistently, the number of cells migrating to the lower chamber in the GNA11 knockdown groups decreased significantly, whereas it increased significantly in the GNA11 overexpression groups ($p < 0.05$; Fig. 5c,d). These results suggest that elevated GNA11 expression can promote the migration ability of ESCC cells, while reduced expression has an inhibitory effect.

Changes in GNA11 expression affected the invasion ability of ESCC cells

As shown in Fig. 6, transwell invasion experiments showed that compared with the control cells, GNA11 knockdown cells exhibited a decreased number of cells that moved to the lower chamber; while GNA11 was overexpressed, the number of invasive cells increased significantly compared with the control cells ($p < 0.001$). These findings suggest that elevated GNA11 expression can enhance the invasion ability of ESCC cells, while reduced expression has an inhibitory effect.

DISCUSSION

In our study, bioinformatics analysis suggested that GNA11 may be implicated in various processes related to the initiation and progression of ESCC. To further elucidate the functional characterization of GNA11 in ESCC, we generated ESCC cell lines with high/low GNA11 expression. *In vitro* experiments demonstrated that GNA11 overexpression could significantly promote the proliferation, migration, and invasion of ESCC cells, and reduce their population dependence. Conversely, downregulation of GNA11



	NES	pvalue	qvalue
ECM−receptor interaction	2.813	7.287e−09	5.216e−07
Focal adhesion	2.656	3.822e−08	9.120e−07
Human papillomavirus infection	2.392	1.887e−06	2.701e−05
MicroRNAs in cancer	2.159	2.580e−04	2.162e−03
Pathways in cancer	1.846	1.685e−03	1.206e−02
PI3K−Akt signaling pathway	2.538	1.196e−07	2.140e−06
Proteoglycans in cancer	1.970	2.478e−03	1.478e−02

Fig. 2 Functional enrichment analysis and signaling pathway prediction. (a) Volcano plot of the DEGs between the high and low GNA11 expression groups. Red indicates upregulated genes, and blue indicates downregulated genes. (b) Analysis of upregulated DEGs and (c) downregulated DEGs by the GO database. (d) Analysis of DEGs by the KEGG database. An adjusted p-value < 0.05 was considered significant. (e) PPI network analysis of GNA11.

had the opposite effect. Previous research has shown elevated levels of anti-GNA11 antibodies in serum and increased GNA11 protein expression in ESCC tissues [11, 12]. Furthermore, studies have indicated that inhibiting Gαq expression can suppress the growth of colorectal cancer cells [18].

Previous research has highlighted the influence of dysregulated proteins on ESCC advancement by regulating signaling, such as HIP1 and MAGE-C3 [19, 20]. Our findings revealed that DEGs associated with GNA11 are linked to multiple pathways involved in cancer development, including the PI3K-Akt, Wnt and Ras protein signaling pathways. GNAQ and GNA11 encode guanine nucleotide-binding protein (G-protein) subunits belonging to the alpha-q subfamily of G-proteins. With 90% amino acid sequence homology, both GNA11 and GNAQ play crucial and overlapping roles in human neoplasia [21, 22]. These G-proteins mediate the signaling of G-protein coupled receptors (GPCRs) by coupling the seven transmembrane domain receptors on the cell membrane to intracellular signaling pathways, including the PI3K-Akt, MAPK pathways and Hippo -YAP pathways [23, 24]. This signaling cascade results in the activation of MAPK

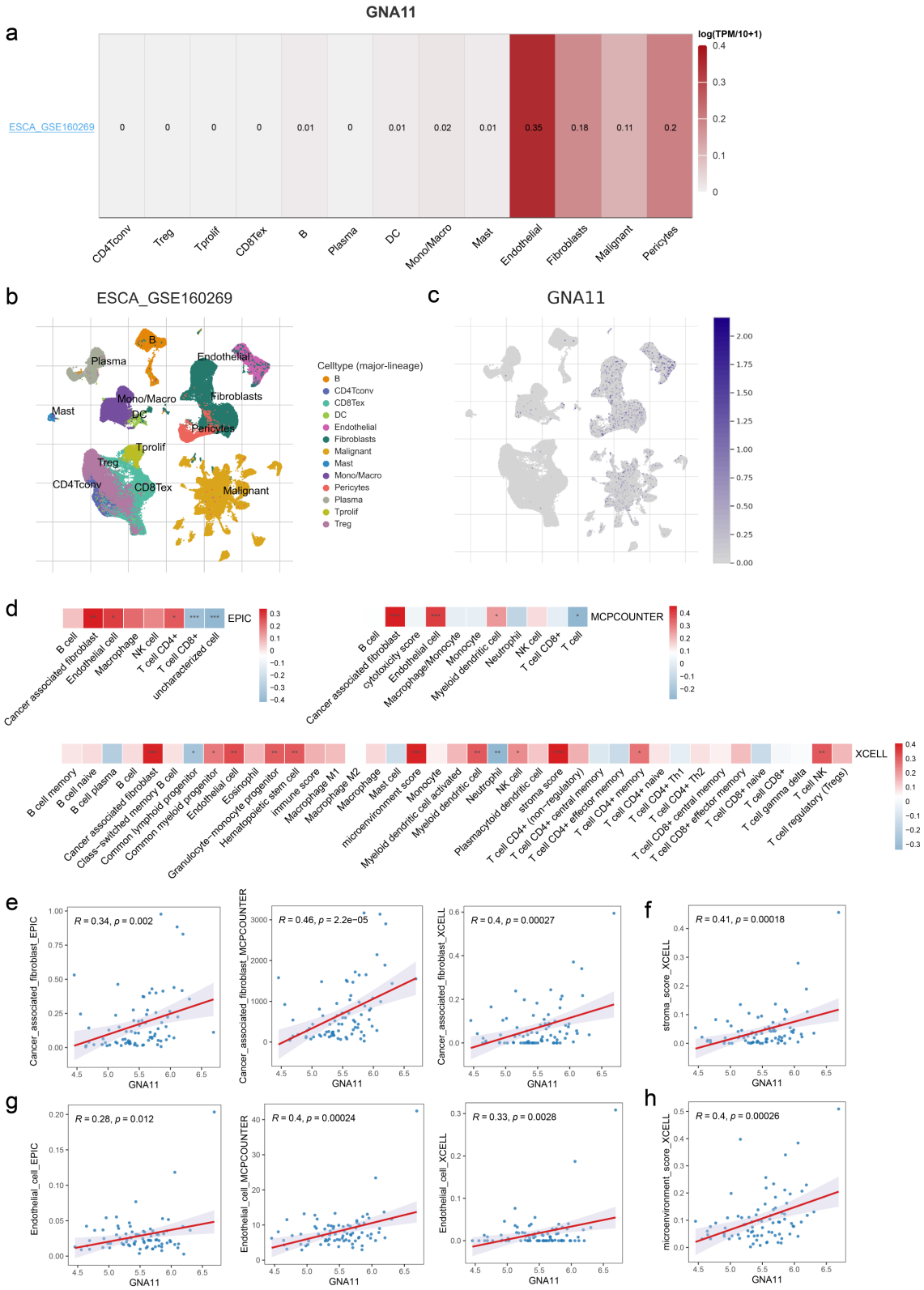


Fig. 3 Research results of GNA11 at the single-cell level. (a) Heatmap displaying the value of GNA11 expression in different cells in the GSE160269 dataset. (b, c) Single-cell cluster map of GNA11 in the GSE160269 dataset. (d) Correlation heatmap of GNA11 and the TME scores calculated by the EPIC, MCPcounter and Xcell algorithms. (e–h) Scatter plot of the correlation of GNA11 with cancer-related fibrocytes, the stroma score, endothelial cells and the microenvironment score.

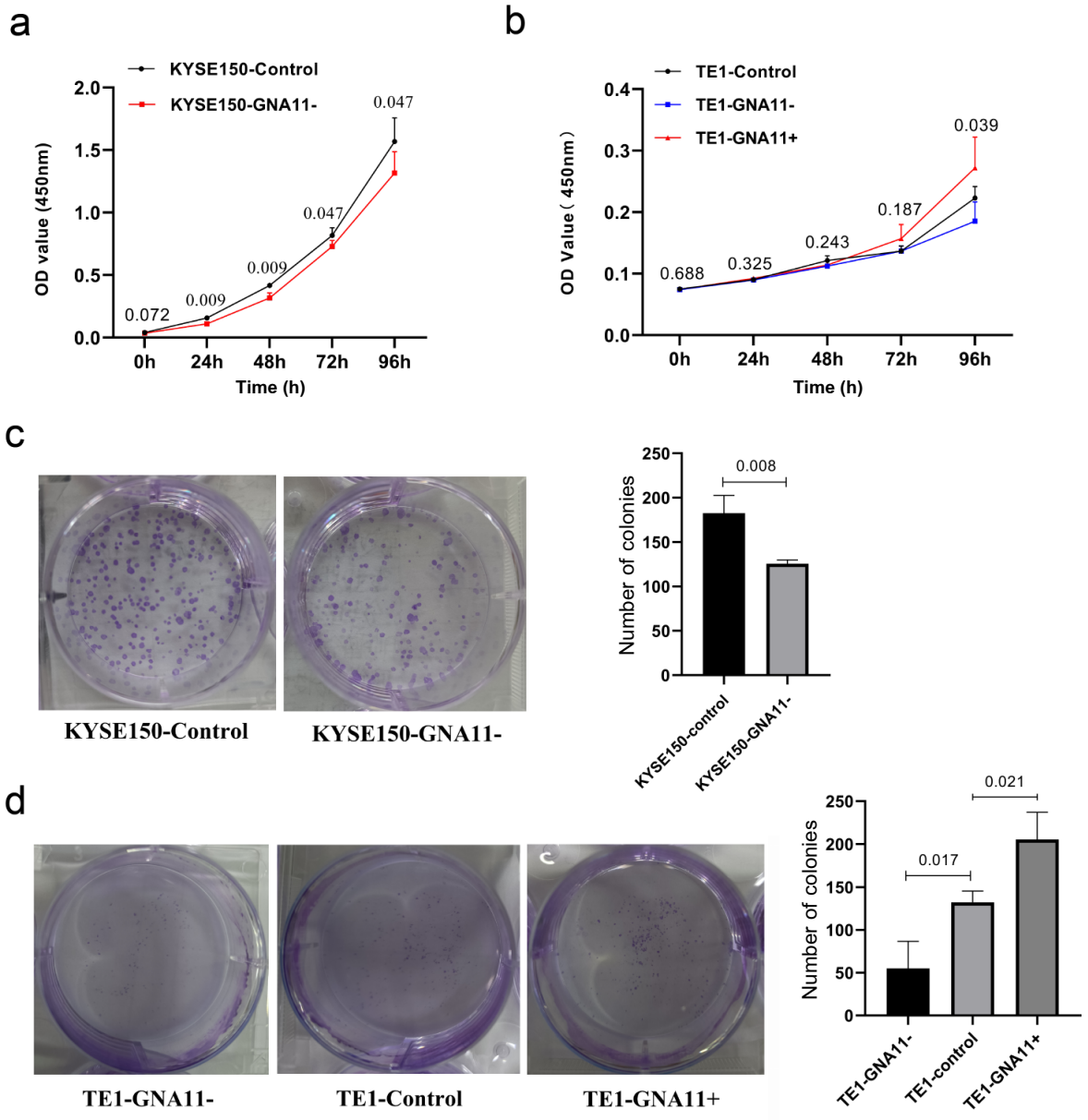


Fig. 4 The expression of GNA11 could affect the proliferation ability of ESCC cells. (a) Cell viability was examined by CCK-8 assay after knockdown or overexpression of GNA11 in KYSE150 cells and (b) TE1 cells. (c) Proliferation ability was detected by colony formation assays after knockdown or overexpression of GNA11 in KYSE150 cells and (d) TE1 cells. The experiments were repeated three times.

pathways, which control cell proliferation, survival, and protein synthesis. Focal adhesion kinase has been identified as a key mediator of Gαq-driven signaling in uveal melanoma (UM), where the Gαq protein encoded by GNAQ/GNA11 promotes PI3K/AKT activation, crucial for the growth of Gαq-driven malignancies [25, 26]. In UM with GNA11 mutation, MAPK activation is significantly altered which is dependent on Ras [27, 28]. Activating mutations in GNAQ/GNA11 result in the dephosphorylation of YAP and TAZ, transcriptional coactivators associated with the Hippo signaling pathways, which promotes their oncogenic activity [23]. Moreover, our study identified enrichment of the DEGs in processes related to extracellular matrix and structure organization, ECM-receptor interaction, focal adhesion, regulation of epithelial mesenchymal transformation, actin filament regulation and other functions associated with cell

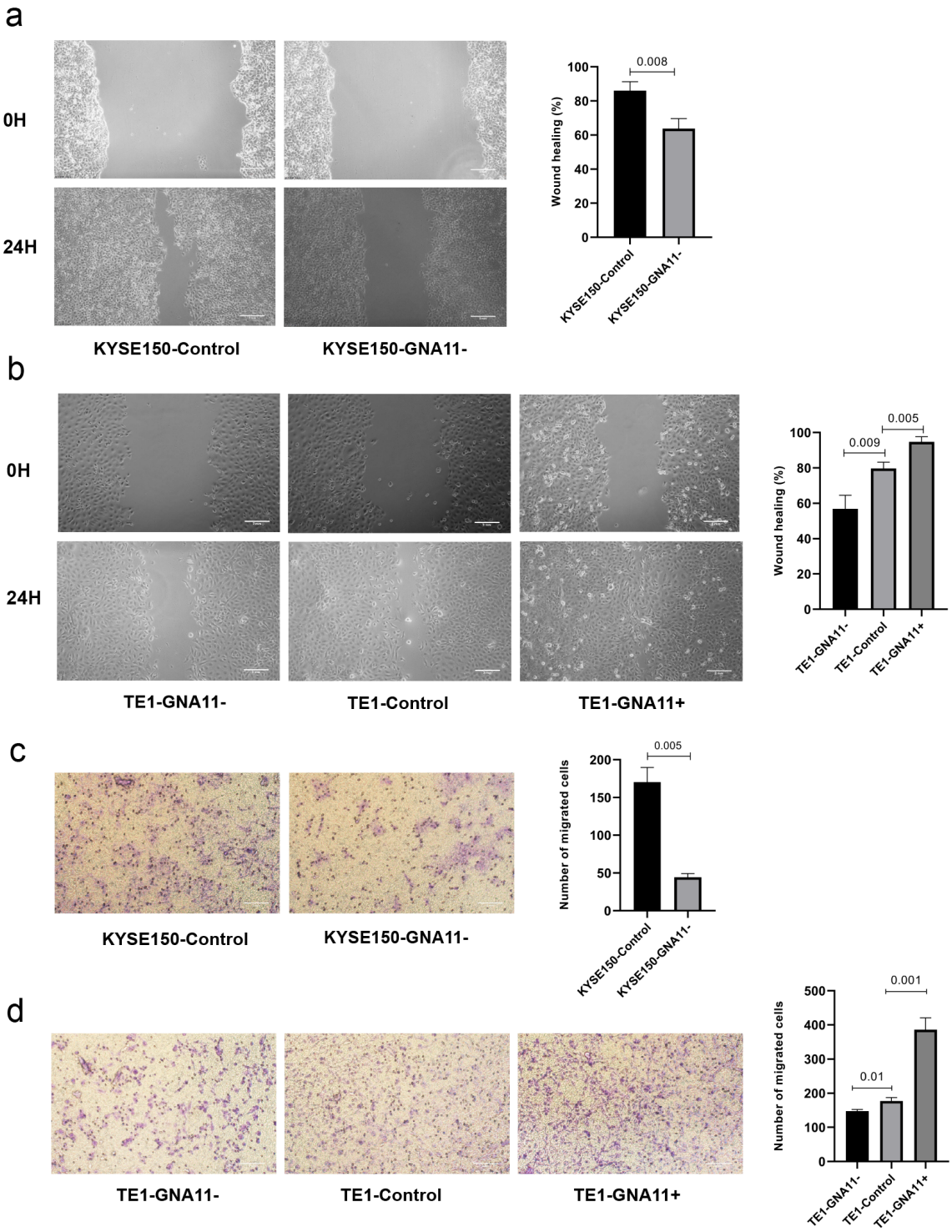


Fig. 5 The expression of GNA11 could affect the migration ability of ESCC cells. (a) The migration ability of KYSE150 and (b) TE1 cells after GNA11 knockdown or overexpression were measured by a wound healing assay. (c) The migration ability of KYSE150 cells and (d) TE1 cells after GNA11 knockdown or overexpression was measured by transwell assays. The experiments were repeated three times. Scale bar, 2 mm.

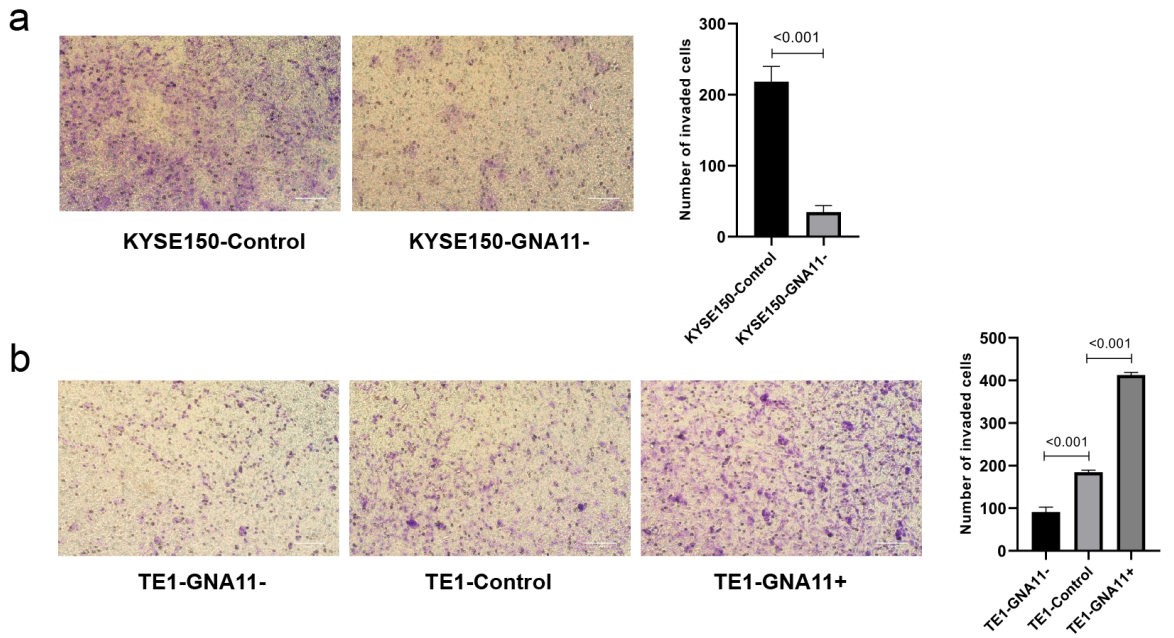


Fig. 6 The expression of GNA11 could affect the invasion ability of ESCC cells. (a) The invasion ability of KYSE150 and (b) TE1 cells after GNA11 knockdown or overexpression was measured by transwell assays. The experiments were repeated three times. Scale bar, 2 mm.

adhesion, migration, differentiation, proliferation and apoptosis. The aggressiveness of cancer cells has been linked to increased traction, high deformability, and altered cell adhesion, with *in vitro* studies suggesting that Gαq/11 may influence melanocyte morphology by regulating actin dynamics [29]. PPI network analysis of GNA11 revealed that its hub factors were mainly involved in signal transduction and G protein-coupled receptor signaling pathways, confirming the functional similarity between GNAQ and GNA11 [30–32]. These findings suggest that GNA11 may play an important role in the numerous signaling pathways involved in stroma-mediated regulation of cell proliferation, adhesion, migration, and differentiation and other cellular processes.

Single-cell RNA sequencing (scRNA-seq) analysis unveiled that GNA11 is predominantly expressed in stromal cells, including endothelial, pericytes, and fibroblasts cells, followed by malignant cells, offering valuable insights into cell behavior within the intricate tumor microenvironment. Stromal cells, such as endothelial cells and fibroblasts cell, need to continuously proliferate and differentiate to maintain tissue homeostasis and promote repair and regeneration. The high expression of GNA11 may help these cells meet these physiological needs, and studies have shown that GNA11 is involved in endothelial cell migration through multiple pathways [33]. In human umbilical cord vein endothelial cells (HUVECs), double knockdown of GNAQ and GNA11 impairs vascular endothelial growth factor A (VEGFA) induced cell migration and inhibits VEGFA [34]. In malignant cells, however, due to factors such as abnormal activation or mutation of cell signaling pathways, the expression and function of GNA11 may be disrupted, resulting in relatively lower expression levels compared to stromal cells or exhibiting a complex expression pattern. This does not imply that GNA11 does not play a significant role in cancer cells. In fact, in certain types of tumors, abnormal expression of GNA11 is one of the crucial factors contributing to tumorigenesis and progression. This study is the first report of GNA11 expression across various cell clusters for cancer research. Tumor immune microenvironment analysis further corroborated these findings, demonstrating a positive correlation between GNA11 expression and CAFs, endothelial cells, the stroma score, and the microenvironment score in ESCC. The tumor microenvironment (TME) comprises malignant cells and stromal cells, with their dynamic crosstalk linked to malignant progression, challenging the conventional belief that malignancy solely arises from malignant cells [35, 36]. CAFs originate from cancer cells and various cell types like bone marrow-derived endothelial, hematopoietic stem, mesenchymal stem, and adipocyte cells, secrete growth factors and cytokines that influence tumor cell proliferation, metastasis, and resistance to multiple therapeutic strategies [35, 37, 38]. CAFs have been observed in cancers such as breast cancer, prostate cancer, and hepatocellular carcinoma, their crosstalk

with cancer cells has been found to affect malignant progression [39, 40]. Therefore, understanding CAFs related factors is important for the exploration of cancer interventions. The above analysis suggests that GNA11 may contribute to cancer promotion through the dynamic crosstalk between the TME and malignant cells, potentially serving as a factor associated with CAFs.

This study systematically investigated the potential role of GNA11 in the progression of ESCC using a bioinformatics system and, for the first time, validated its functional characterization in ESCC through *in vitro* functional experiments. While this study contributes to clarifying the relationship between GNA11 and ESCC, there are several limitations that warrant consideration. Firstly, GNA11 mutations are frequently observed in the progression of various cancers, such as melanoma and esophageal adenocarcinoma. However, mutation-related data were not available in this study for further analysis. Secondly, the study solely focused on verifying the expression and function of GNA11 in ESCC cancer cells, without exploring its potential role in other cell types, such as stromal cells. Future research addressing the relationship between GNA11 mutations and ESCC, as well as investigating the function of GNA11 across diverse cell populations, is eagerly anticipated.

In summary, the overexpression of GNA11 can enhance the proliferation, migration, and invasion of ESCC cells and may play a role in "stroma-cell" and "stroma cell-malignant cell" interactions through the regulation of cancer-related fibrocytes, the PI3K-Akt signaling pathway, ECM-receptor interaction, focal adhesion, etc. Our findings indicate that GNA11 plays an important role in the occurrence and progression of ESCC. Additionally, it is identified as a potential therapeutic target.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.061. Supplementary tables and raw datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

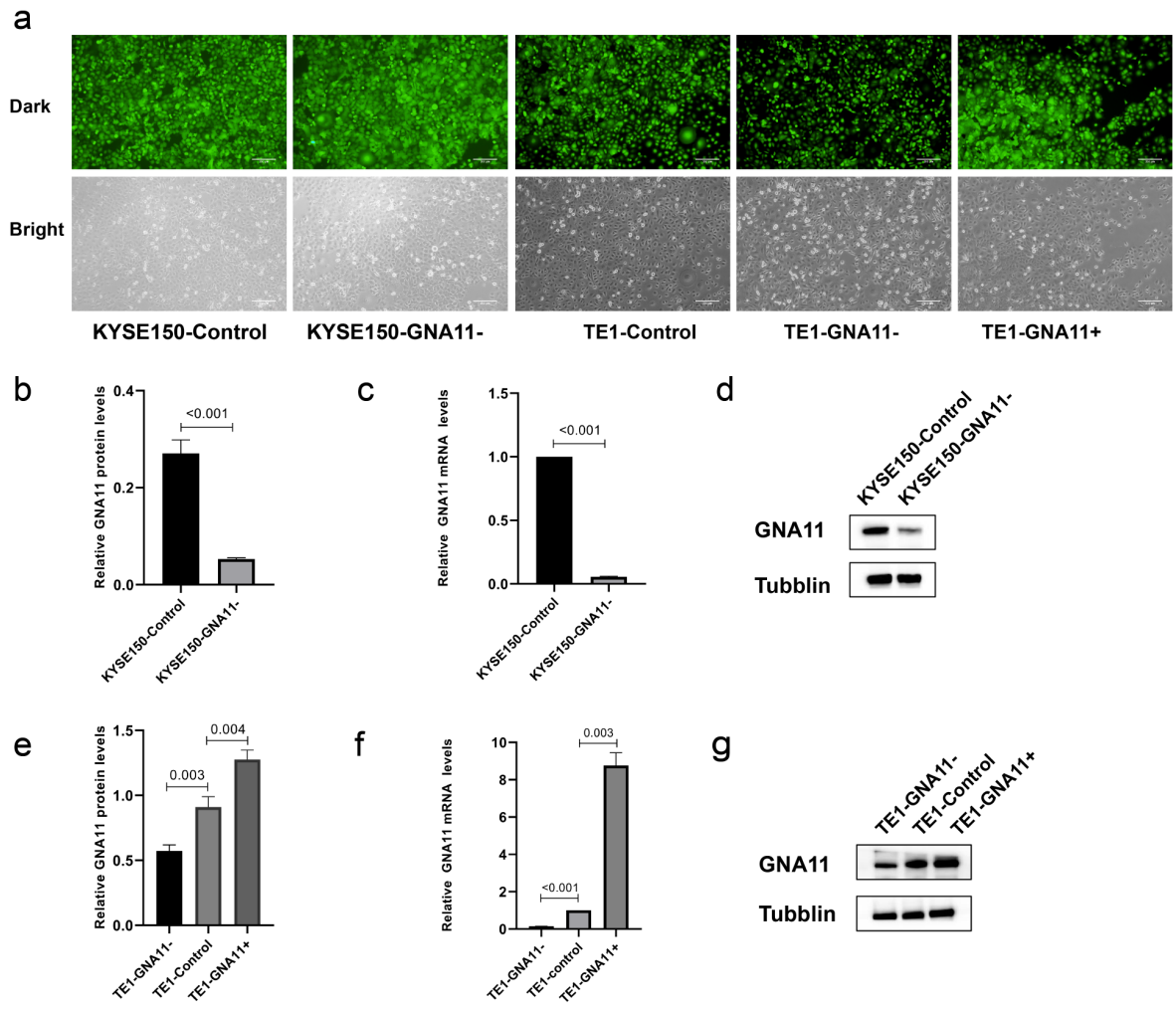

Fig. S1 The mRNA and protein expression of GNA11 in ESCC cells. (a) The expression efficiency of GFP was observed by fluorescence microscope after ESCC cells transfected with lentivirus after 96 h. (b–d) Knockdown and overexpression of GNA11 in KYSE150 and (e–g) TE1 cells. Scale bar, 200 μm; * $p < 0.001$.