


## ORIGINAL ARTICLE

# GPR12 inhibits migration and promotes apoptosis in esophageal cancer and hypopharyngeal cancer cells

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## Abstract

**Background:** G protein-coupled receptor 12 (GPR12) is an orphan receptor with no confirmed endogenous ligands. It plays important roles in both physiological and pathological conditions such as neurogenesis and neural inflammation. However, it remains unclear whether GPR12 regulates carcinogenesis and progression in head and neck squamous cell carcinoma (HNSCC), such as esophageal cancer (EC) and hypopharyngeal cancer (HC).

**Methods:** The Cancer Genome Atlas (TCGA) database was applied to explore the expression of GPR12. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression of GPR12 in cancer tissues. Wound healing and transwell assays were carried out to verify the effect of GPR12 on cell migration. Flow cytometric analysis and caspase-Glo 3/7 assay were carried out to verify the influence of GPR12 on cell apoptosis. Western blotting was used to measure the expression of proteins related to migration and apoptosis.

**Result:** The qRT-PCR analyses showed that the expression of GPR12 decreased in EC and HC than that in their paired adjacent normal tissues. Wound healing assay and transwell assay demonstrated that GPR12 inhibited tumor cell migration. Flow cytometry analysis and Caspase-Glo 3/7 Assay suggested that GPR12 promoted apoptosis. The mechanism of GPR12 may function via modulating caspase-7, E-cadherin, and  $\alpha$ -catenin in EC and HC cells.

**Conclusion:** In conclusion, GPR12 induced apoptosis by activating caspase-7 and inhibited migration through epithelial-to-mesenchymal transition (EMT) in EC and HC. Our findings demonstrated that GPR12 as a potential tumor suppressor mediated cell migration and apoptosis in EC and HC.

## KEYWORDS

Apoptosis, epithelial-to-mesenchymal transition, esophageal cancer, GPR12; migration

## INTRODUCTION

Esophageal cancer (EC) is an upper gastrointestinal tumor whose pathological form is mainly squamous cell carcinoma. EC is one of the most common cancers in the world and its incidence accounts for ~70% of the world.<sup>1,2</sup> Hypopharyngeal cancer (HC) is located at the intersection of the upper digestive tract and the airway whose pathological form is also

mainly squamous cell carcinoma. HC accounts for 3% of head and neck cancers, but it is difficult to diagnose and the prognosis is very dismal, with a 5-year overall survival rate of only ~30%–35%.<sup>3,4</sup> Multiple primary carcinoma (MPC) of the hypopharynx and esophagus are the most common and occur at the same time or at different times.<sup>5–8</sup> The cause of MPC is still unclear, and the “regional carcinogenesis” of tumor polycentric origin is a relatively reasonable mechanism.<sup>9</sup> The

hypopharynx and the esophagus are adjacent, and the mucosal epithelium is squamous epithelium, which are stimulated by common carcinogens to form independent and separated precancerous lesions or malignant tumors. These two types of cancers are usually poorly differentiated and develop lymph node metastasis early. Although the development of surgical procedures and radiotherapy techniques in recent years has greatly improved the survival rate and quality of life in these patients, the 5-year survival rate is still very low, especially for MPC. There is an urgent need to explore in-depth the pathogenesis of EC and HC and seek strategies to improve the prognosis of patients and increase the long-term survival rate.

G protein-coupled receptor 12 (GPR12) is an orphan receptor that belongs to the class A family of G protein-coupled receptors (GPCRs). The human sequence of GPR12 contains 334 amino acids that shares ~60% of sequence identity with those of GPR3 and GPR6, suggesting similar ligands and functions may exist for them.<sup>10</sup> In the past few years, studies have found that GPR12, GPR3, and GPR6 are abundant in the nervous system<sup>11,12</sup> and are closely related to the pathogenesis of many central nervous system (CNS) diseases.<sup>13,14</sup> It has been certificated that GPR3 and GPR12 can maintain meiotic arrest in rodent oocytes.<sup>15</sup> The abundance and effects of GPR12 vary in different cell lines, although its function and mechanism are still unclear in EC and HC. In the present study, we sought to explore the possible functions and mechanism of GPR12 in EC and HC.

Based on the findings here, we determined the expression of GPR12 and its clinical and prognostic relevance in EC and HC. We also investigated its activities in vitro and characterized the potential underlying mechanisms of action, suggesting that GPR12 may function as a tumor suppressor in EC and HC.

## METHODS

### Patient specimens

The cDNA array of 11 samples of EC was obtained from Outdo Biotech. Samples were obtained from 18 patients with HC who underwent surgery at the Qilu Hospital of Shandong University. No patients received any chemotherapy before surgery. All patients were randomly selected. Samples were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. All clinical specimens were used to determine the GPR12 expression by quantitative real-time polymerase chain reaction (qRT-PCR). The clinical information of patients was retrieved from patients' medical records. All patients signed the informed consent when admitted. This study was approved by the Institutional Ethics Committee at Qilu Hospital.

### Access to public data sets

The expression profile of GPR12 was analyzed using RNA sequencing (RNA-seq) data of cancer tissues and respective

adjacent normal mucosae in The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>) and Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). A Gene Set Enrichment Analysis (GSEA, <http://www.gsea-msigdb.org/gsea/index.jsp>) was performed as previously described.<sup>16</sup>

### Cell lines and culture

The human EC cell line Eca109 was preserved in our lab. The HC cell line FaDu was obtained from American Type Culture Collection. Eca109 cells were maintained in DMEM medium (BasalMedia) containing 10% fetal bovine serum (FBS) (Lanzhou Minhai Bio-Engineering). FaDu cells was cultured in MEM medium (BasalMedia) with 10% FBS. All cells were maintained in a humidified incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ .

### RNA extraction and qRT-PCR

Total RNAs were extracted from tissues and cells by TRIzol reagent (Sigma-Aldrich). Approximately  $1\ \mu\text{g}$  of RNA of each sample was reversely transcribed to cDNA by the reverse transcription kit (Vazyme). The qRT-PCR was then carried out using a ChamQ Universal SYBR qPCR Master Mix (Vazyme). The human GPR12-specific primers used were 5'-CTGGGACATTGTCTTGTGTACC-3' and 5'-CAGGCTGCCTATTAGCAGGAA-3'. The universal  $\beta$ -actin-specific primers 5'-GAAGAGCTACGAGCTGCCTGA-3' and 5'-CAGACAGC ACTGTGTTGGCG-3' were used as control primers. Relative quantification was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method.

### Plasmid and cell transfection

GPR12 plasmid was purchased from Vigenebio. Transiently transfection of cells was performed using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's instruction. Cells were plated in 6-well plates at a concentration of  $2.5 \times 10^5$  cells/mL for 24 hours. When the cell confluence reached 50%–70%, transfection was performed. We diluted 5  $\mu\text{L}$  Lipofectamine 3000 reagent and 2  $\mu\text{g}$  plasmids into 125  $\mu\text{L}$  Opti-MEM (Thermo Fisher) medium, respectively. Next, 5  $\mu\text{L}$  P3000 reagent was added into the diluted plasmid solution. After briefly vortexing, the mixture was incubated at room temperature for 5 minutes. The diluted plasmid solution was then added to the diluted Lipofectamine 3000 reagent. After 15 minutes incubation at room temperature, the plasmid-lipid complex was added to cells. Forty-eight hours after transfection, qRT-PCR and western blot were used to verify the expression of GPR12.

### Transwell assay

Transwell chambers were used to detect the invasion of cells. Approximately  $1 \times 10^5$  transiently transfected cells were cultured in the upper chamber with serum-free medium, while

medium containing 20% FBS was added to the lower chamber. Cells were maintained at 37°C for 24 hours and were then washed with PBS and fixed with methanol. After that, 0.1% crystal violet stain solution (Solarbio) was used to stain the cells. Finally, cells were photographed under a microscope, and stained cells were counted.

### Wound healing assay

Transiently transfected cells were plated in 6-well plates at a concentration of  $3 \times 10^5$  cells/mL for 24 hours and then scratched in wells with a pipette tip. After being washed by phosphate buffer saline (PBS), MEM, or DMEM medium with 1% FBS was added to plates. Pictures of the scratches were taken every 6 hours to calculate the scratch healing area.

### Cell counting kit-8 assay

Approximately  $5 \times 10^3$  transiently transfected cells in 100  $\mu$ L medium were maintained in 96-well plates. After 24, 48, and 72 hours incubation, 10  $\mu$ L cell counting kit-8 assay (CCK8) solution was added to each well. After 2 hours of additional incubation, the optical density (OD) value at 450 nm was measured with microplate reader.

### Flow cytometric analysis

Apoptosis of cells was determined using an Annexin V-FITC/PI Apoptosis Detection Kit (Bestbio). Transient cells were plated in 6-well plates at a concentration of  $2 \times 10^5$  cells/mL for 48 hours. The cells were then harvested by ethylenediamine-tetraacetic acid (EDTA)-free trypsin and washed with PBS. After that, the cells were resuspended with binding buffer and stained with fluorescein isothiocyanate (FITC) annexin V and propidium iodide (PI), respectively. The entire incubation process was carried out in the dark. Results were quantified using CytEpt v2.0 (Beckman Coulter).

The cells' cycle was detected by cell cycle detection kit (Bestbio). Transiently transfected cells were plated in 6-well plates at a concentration of  $2 \times 10^5$  cells/mL for 48 hours. After the cells were digested with trypsin, they were fixed with 75% ice-cold ethanol and stored at -20 for 1 hour. The cell suspension was then incubated at 37°C for 30 minutes after adding 20  $\mu$ L Rnase A reagent. After discarding the supernatant, we resuspended the cells with 0.5 mL PI staining buffer and incubated the cells for 15 minutes at room temperature in the dark. The cell cycle was then immediately detected via a flow cytometer (Beckman Coulter, Cytotflex S).

### Caspase-Glo 3/7 assay

Cells were plated in 96-well plates at a concentration of  $5 \times 10^4$  cells/mL for 24 hours. Caspase-Glo 3/7 Reagent

(Promega) was added to each well. After being gently mixed with a plate shaker, cells were incubated at room temperature. Finally, luminescence of each sample was measured in a plate-reading luminometer.

### Western blotting

Cells were harvested by trypsin and lysed with an ultrasonic lyser in the RIPA buffer (Thermo Fisher) with protease inhibitors (Sigma). The supernatant was analyzed for protein content using a bicinchoninic acid (BCA) protein assay kit (Merck). The heat-denatured samples were separated on 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% milk-tris-buffered saline plus tween 20 (TBST) and incubated with primary and secondary antibodies. The grayscale values of protein bands were analyzed using ImageJ software (National Institutes of Health). The following antibodies were used: a rabbit anti-Caspase-7 polyclonal antibody (1:1000 dilution; Proteintech), rabbit monoclonal antibodies against E-cadherin, N-cadherin, vimentin, and  $\alpha$ -catenin (1:1000 dilution; Abcam), a goat anti-mouse IgG H&L (DyLight 680 4X PEG Conjugate) (1:10,000 dilution; Cell Signaling Technology), a goat anti-rabbit IgG H&L (DyLight 800 4X PEG Conjugate) (1:10,000 dilution; Cell Signaling Technology), and a mouse anti- $\beta$ -actin monoclonal antibody (1:1000 dilution; Sigma) as the loading control.

### Statistical analyses

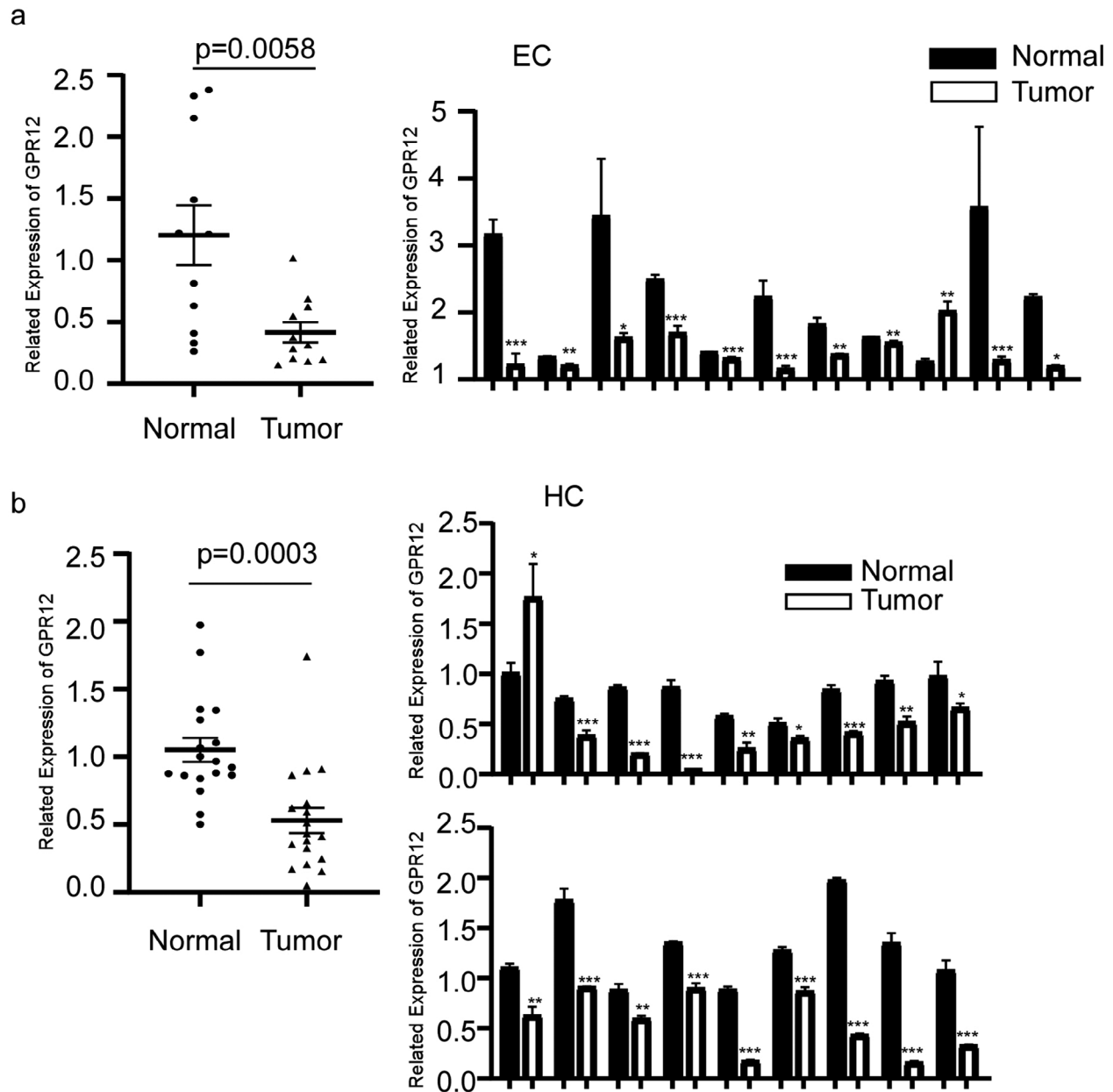
Paired or unpaired Student's *t*-tests were used for continuous variables. The Fisher exact test and  $\chi^2$  tests were used for categorical comparisons. Survival analyses were evaluated by the Kaplan–Meier method. All tests and reported *p* values were two-sided, and *p* < 0.05 was defined as statistically significant. Statistical analyses were performed with GraphPad Prism7 (GraphPad).

## RESULTS

### Expression of GPR12 in EC and HC

Because GPR12 had not been investigated in EC and HC previously, the expression pattern of GPR12 in these carcinoma tissues compared to that in adjacent normal tissues using clinically resected samples were detected. GPR12 was significantly decreased in 17 of 18 HCs (Figure 1(a)) and 10 out of 11 ECs (Figure 1(b)), compared to that in corresponding adjacent normal tissues.

To further validate these results, we analyzed the expression profile of GPR12 in the TCGA data sets of EC (Figure 2(a)), head and neck cancer (HNC) (Figure 2(b)), laryngeal cancer (LC) (Figure 2(c)), oral cancer (OC) (Figure 2(d)), and



**FIGURE 1** GPR12 decreased in EC and HC tissues. (a) Relative expression level of GPR12 in 11 pairs of EC tissues and adjacent normal tissues measured by qRT-PCR ( $n = 11$ , paired  $t$ -test). (b) Relative expression level of GPR12 in 18 pairs of HC tissues and adjacent normal tissues measured by qRT-PCR ( $n = 18$ , paired  $t$ -test). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

tongue cancer (TC) (Figure 2(e)), and in the GEO data sets of GSE 30784 (Figure 2(f)) and GSE84957 (Figure 2(g)). GPR12 was significantly decreased in cancer tissues than in adjacent normal tissues. Taken together, these results demonstrated that GPR12 was decreased in EC and HC.

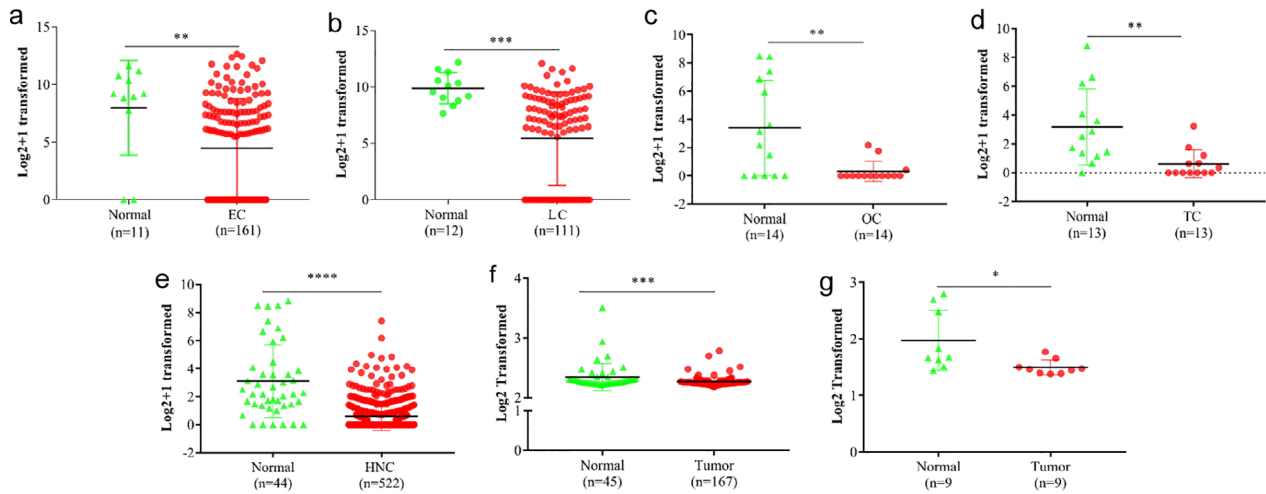
### GPR12 inhibited cells migration

To further examine the functional relevance of GPR12 EC and HC, the activity of GPR12 in vitro in Eca109 and FaDu cells using wound healing assay and transwell assays was investigated. As shown in Figure 3(a), 48 hours after the scratch, the

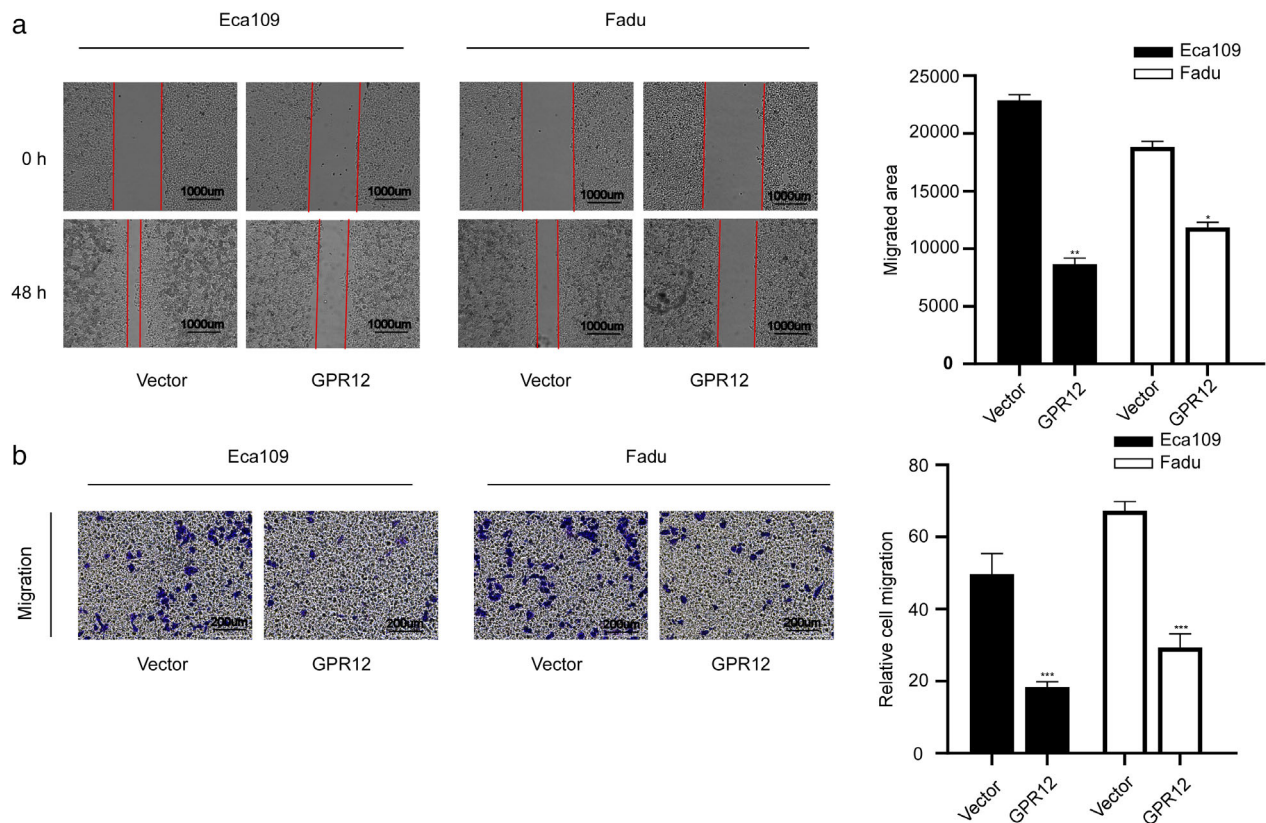
migration area was significantly reduced in Eca109 and FaDu cells transferred with GPR12 compared with the control group ( $p < 0.05$ ). Moreover, the migration cells were also reduced by overexpression of GPR12 compared with the control group ( $p < 0.05$ ) (Figure 3(b)). In conclusion, GPR12 could inhibit Eca109 and FaDu cells migration.

### GPR12 promoted cells apoptosis

Because apoptosis plays an important role in tumor regression, we explored whether GPR12 could mediate this process. Overexpression of GPR12 promoted apoptosis of



**FIGURE 2** Expression profile of GPR12 in the TCGA and GEO data sets. (a–e) the expression of GPR12 was significantly decreased in the TCGA data sets of EC, LC, TC, OC, and HNC cancer tissues. (f) and (g) The expression of GPR12 was significantly decreased in the GEO data sets of cancer tissues. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

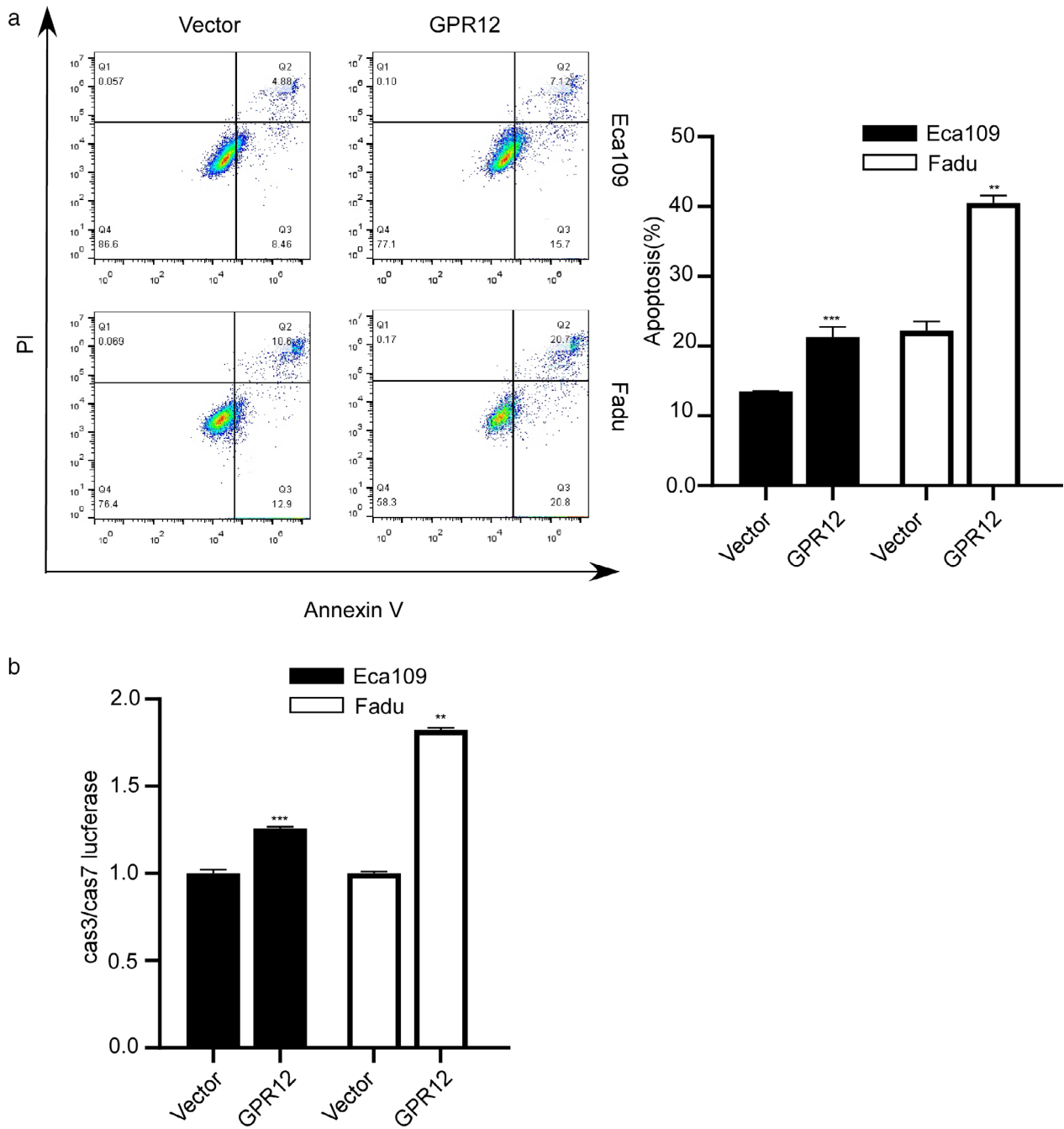


**FIGURE 3** GPR12 inhibited the cell migration. (a) GPR12 inhibited the wound healing of Eca109 and FaDu cells; the area of migration cells transfected with GPR12 is smaller. (b) GPR12 inhibited the chamber migrating of Eca109 and FaDu cells; the amount of migration cells transfected with GPR12 is fewer. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Eca109 and FaDu cells compared to the control group, the effect is more pronounced in FaDu cells ( $p < 0.05$ ) (Figure 4 (a)). GPR12 also increased the Caspase-Glo 3/7 luciferase activity in Eca109 and FaDu cells compared to the control group ( $p < 0.05$ ) (Figure 4(b)).

### Effect of GPR12 on cell cycle and proliferation

Results shown in Figure 5 indicate that GPR12 had little effect on cell cycle of Eca109 cell (Figure 5(a)) and FaDu cell (Figure 5(b)). Meanwhile, there was no significant difference



**FIGURE 4** GPR12 promoted the cell apoptosis. (a) GPR12 promotes the percentage of apoptosis in Eca109 and FaDu cells. (b) GPR12 increased the Caspase-3/7 luciferase activity in Eca109 and FaDu cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

in proliferation between Eca109 and FaDu cells transfected with GPR12 and the control group. (Figure 5(c) and (d)).

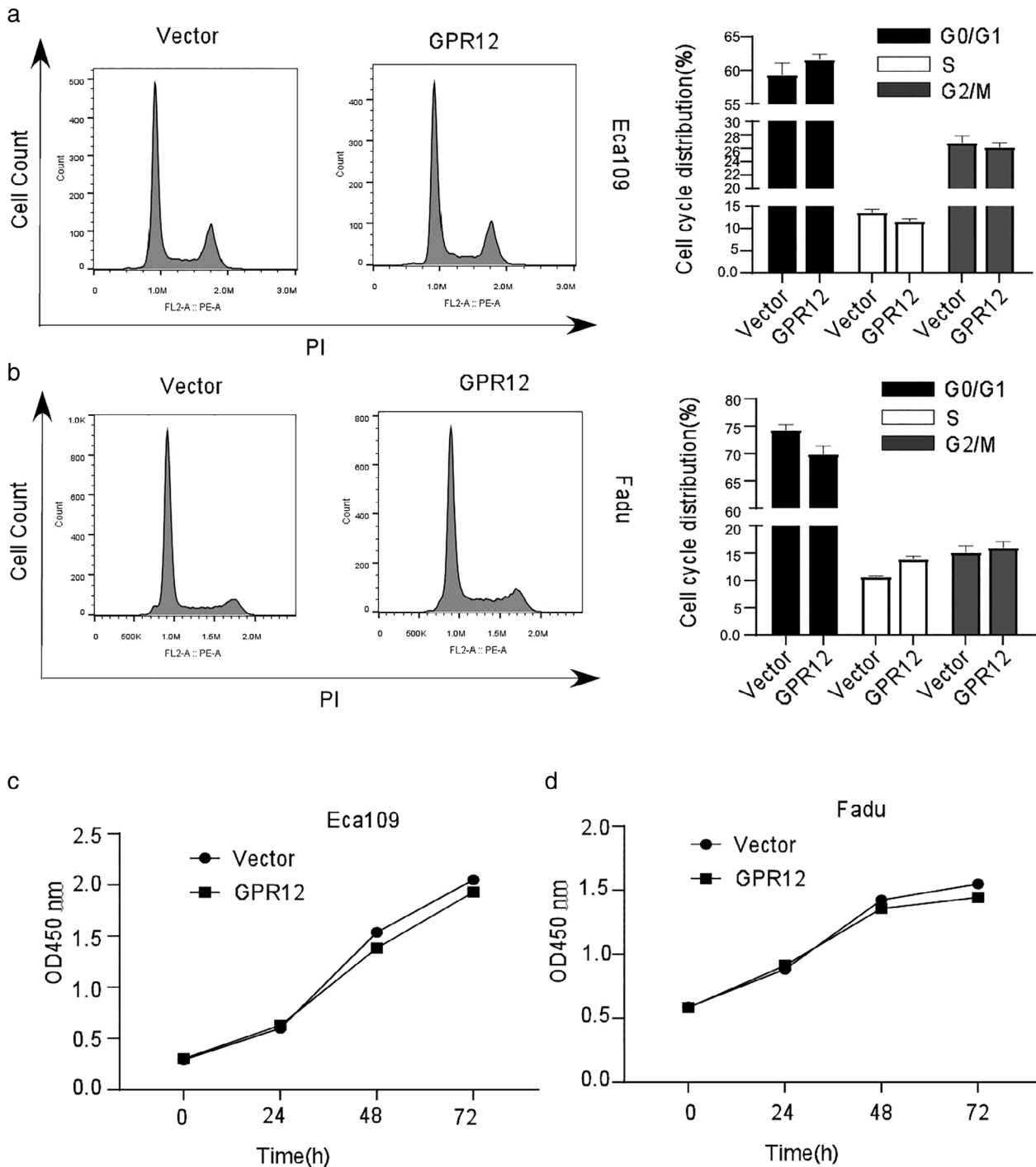
### GPR12 function by interacting with key gene sets associated with tumor progression

The possible mechanisms underlying GPR12's role in EC and HC were explored. A GSEA was performed using respective TCGA data sets. GPR12 was negatively correlated

with several hallmark gene sets that were closely related to tumor progression of cells migration and apoptosis, such as epithelial-mesenchymal transition (EMT) (Figure 6).

### GPR12 affected the proteins of EMT and caspase-7

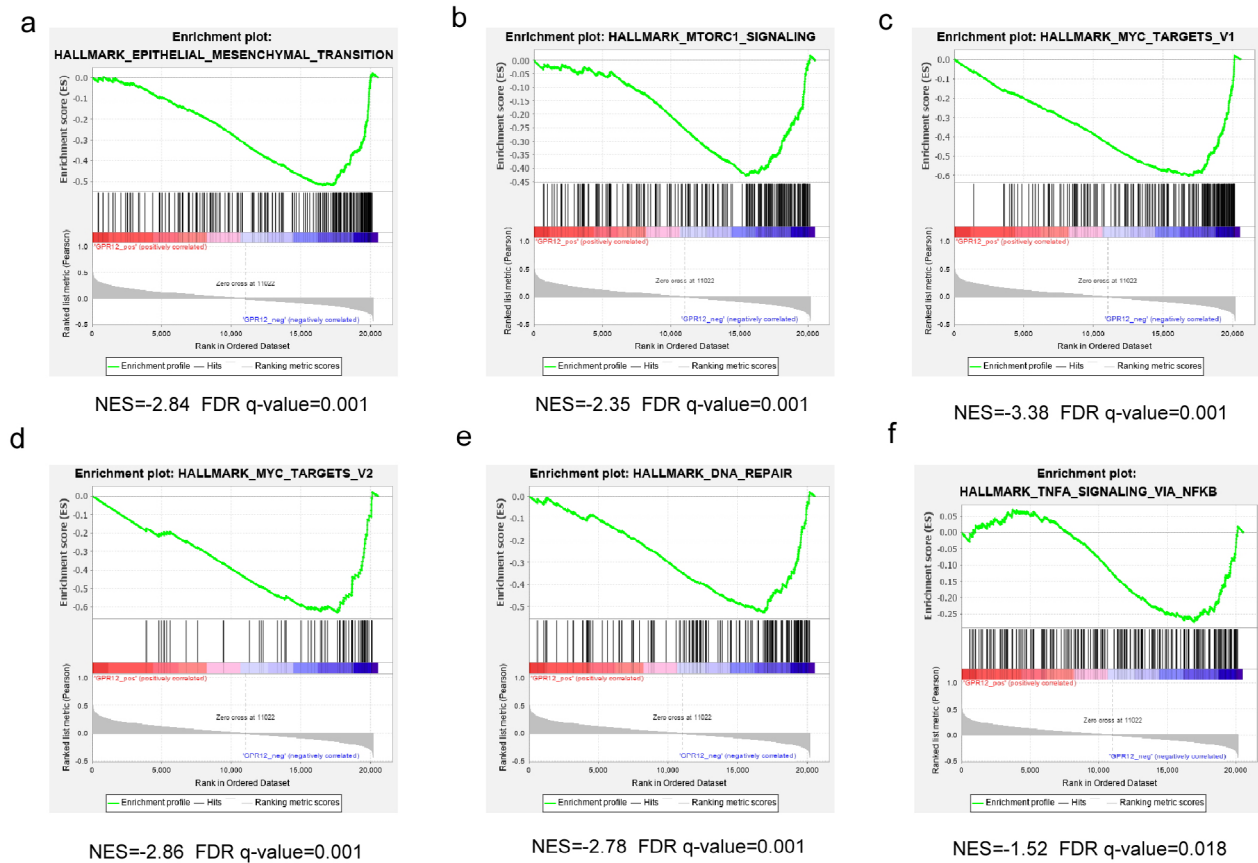
To explore the mechanism of GPR12 on cells migration, the expression of EMT-related proteins was measured. Forty-



**FIGURE 5** The effect of GPR12 on cell cycle and proliferation in vitro. (a) and (b) The expression of GPR12 had little influence on Eca109 and FaDu cells cycle compared with negative control cells ( $n = 3$ ). (c) and (d) GPR12 did not affect Eca109 and FaDu cells proliferation compared with negative control cells ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

eight hours after transiently transfected with GPR12, the expression of E-cadherin and  $\alpha$ -catenin were increased in Eca109 and FaDu cells transferred with GPR12 compared to control group ( $p < 0.05$ ) (Figure 7(a) and (b)). However, the expression of N-cadherin and vimentin did not change a lot ( $p < 0.05$ ) (Figure 7(c) and (d)). The results indicated that GPR12 regulated cell migration through regulating E-cadherin and  $\alpha$ -catenin.

Meanwhile, to further study the reason of cell apoptosis induced by GPR12, the expression of cleaved caspase-7 was also examined. Overexpression of GPR12 increased the cleaved caspase-7 in Eca109 and FaDu cells (Figure 7(e)), which indicated that GPR12 promoted Eca109 and FaDu cells apoptosis by increasing the cleaved caspase-7. Collectively, these results confirmed that GPR12 probably inhibited cells migration through



**FIGURE 6** The analysis GSEA indicated GPR12 negatively correlated with the key gene sets of tumor progression. (a) GPR12 was negatively correlated with HALLMARK\_EPITHELIAL\_MESENCHYMAL\_TRANSITION. (b) HALLMARK\_MTORC1\_SIGNALING. (c) HALLMARK\_MYC\_TARGETS\_V1. (d) HALLMARK\_MYC\_TARGETS\_V2. (e) HALLMARK\_DNA\_REPAIR. (f) HALLMARK\_TNFA\_SIGNALING\_VIA\_NFKB

EMT progression and promoted cells apoptosis through caspase-7.

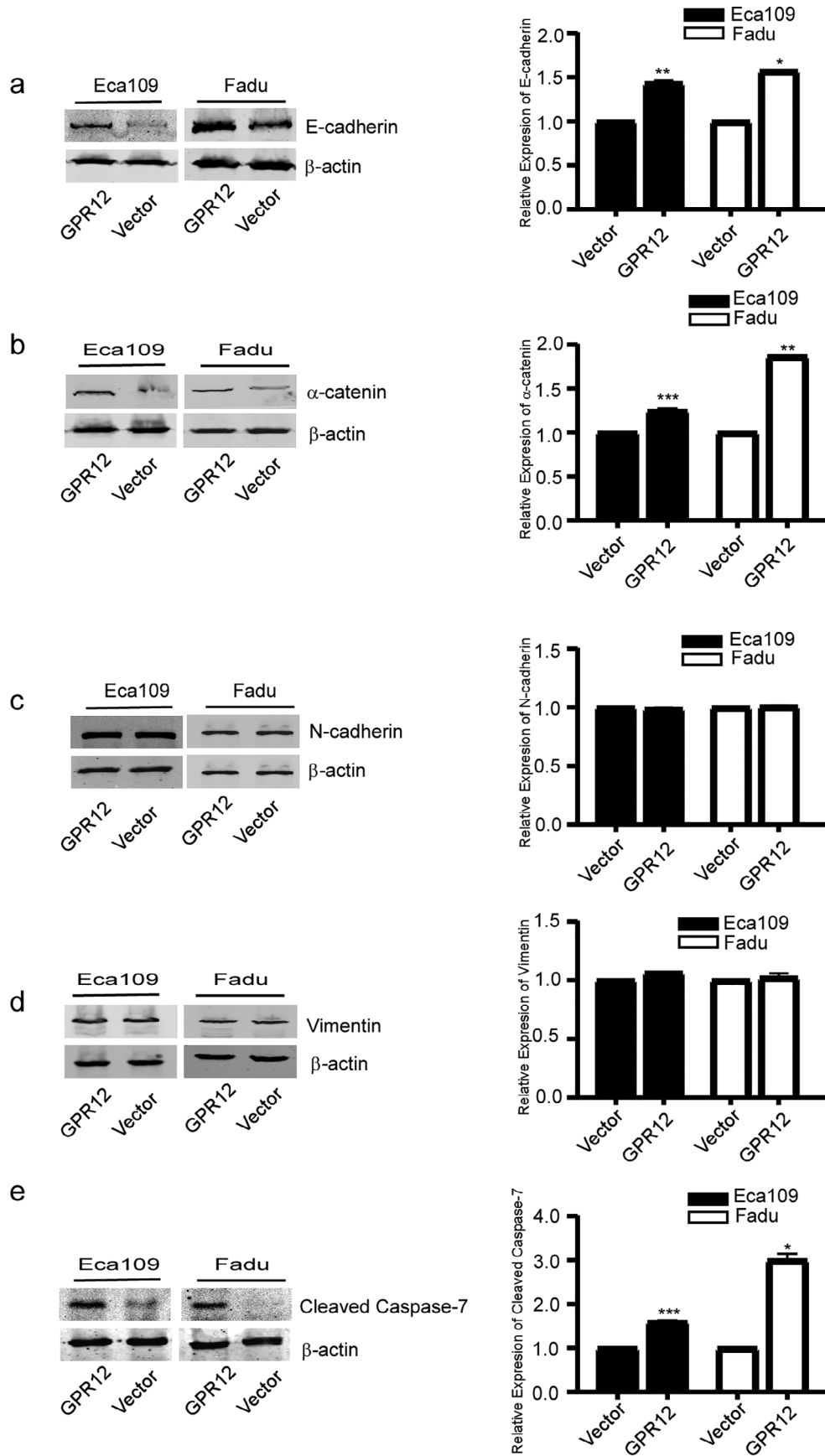
## DISCUSSION

In the current study, because the number of HC patients in the TCGA database was scarce, the expression profile analysis of GPR12 in HC could not be performed. However, the expression profile of GPR12 in LC, OC, and TC, which all belong to HNC and pathological forms are all squamous cell carcinoma originated from the mucosa, were explored. GPR12 was significantly decreased in cancer tissues compared to adjacent normal tissues. GPR12 was also significantly decreased in cancer tissues compared to adjacent normal tissues of HC and EC patient specimens. These results indicated that GPR12 was related to the pathogenesis of EC and HC, and the function study of GPR12 on EC and HC should be performed.

EMT refers to the transition of epithelial to mesenchymal phenotypes in cells. It not only plays a key role in the developmental process, but also participates in other processes, such as wound healing, organ fibrosis, and carcinogenesis,<sup>17–19</sup> and could increase the ability of cells to metastasize and invade.<sup>20–22</sup> A hallmark of EMT is the downregulation of

E-cadherin to reinforce the destabilization of adherens junctions. Meanwhile, the downregulation of E-cadherin is often accompanied by the upregulation of mesenchymal N-cadherin, which results in a “cadherin switch” that alters cell adhesion.<sup>23,24</sup> In addition, EMT is also manifested by decrease of  $\alpha$ -catenin and increase of vimentin expression. After this transition, tumor cells lose their epithelial phenotypes and gradually turn into mesenchymal phenotypes, which lead to an increase in cell migration and invasion ability.<sup>25</sup> In the current study, we found that the expression of GPR12 was significantly decreased in EC and HC compared to that in respective adjacent normal tissues. Overexpression of GPR12 impeded tumor cell migration, whereas higher expression of GPR12 was negatively correlated with hallmark gene set epithelial–mesenchymal transition. Overexpression of GPR12 dramatically upregulated protein expression of E-cadherin and  $\alpha$ -catenin in EC and HC. These observations clearly demonstrated that GPR12 could inhibit the migration of Eca109 and FaDu cells through inhibiting EMT progression, although the underlying mechanism remained to be elucidated. There have been numerous studies on GPR12 in neurology,<sup>26</sup> however, few such studies focus on tumors. Our research shows that GPR12 could be a potential therapeutic target for regulating EMT and cancer migration.





**FIGURE 7** GPR12 inhibited EMT progression and induced cleaved caspase-7. (a) and (b) GPR12 increased E-cadherin and α-catenin in Eca109 and FaDu cells. (c) and (d) GPR12 had no influence on N-cadherin and vimentin in Eca109 and FaDu cells. (e) GPR12 increased the cleaved caspase-7 related with apoptosis in Eca109 and FaDu cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Apoptosis is a kind of programmed cell death, which was first proposed in 1972.<sup>27</sup> Apoptosis is a complex process, involving the activation and expression of many genes. In previous studies, it has been proven that mitochondria are not only the center of cellular respiratory chain and oxidative phosphorylation, but also the playmarker of apoptosis.<sup>28</sup> Cytochrome C released into the cytoplasm can interact with apoptosis-related factor 1 (Apaf-1) to form multimers and promote caspase-9 to form apoptotic bodies. After being activated, caspase-9 can further activate other caspases, such as caspase-7, thereby inducing cell apoptosis.<sup>29</sup> In our current study, we confirmed that overexpression of GPR12 could promote tumor cell apoptosis and increase the expression of cleaved caspase-7. Meanwhile, in the previous studies, it was demonstrated that G protein orphan receptors, such as GPR3, were positively correlated with the pathogenesis of many neurodegenerative diseases such as Alzheimer's disease.<sup>13</sup> It is known that nerve cell apoptosis and mitochondrial dysfunction are the main pathological features of Alzheimer's disease.<sup>30</sup> Therefore, we suspect that GPR12 may promote cell apoptosis through the mitochondrial pathway.

Although our research has identified GPR12 as a potential factor in the pathogenesis of EC and HC, there are some limitations that need to be resolved in future researches. First, we did not confirm the tumor-suppressor role of GPR12 in vivo, which might be quite different from in vitro observations, considering the complex interactions between tumor and the microenvironment. In addition, although we have revealed the role of GPR12 in the migration and apoptosis of EC and HC, the underlying mechanism requires further in-depth research to reveal the regulation of GPR12 for caspase-7, E-cadherin, and  $\alpha$ -catenin. Our study also indicated that GPR12 has almost no effect on the proliferation and cycle of Eca109 and FaDu cells.

In summary, the current study presents a novel finding that the expression of GPR12 is lower in EC and HC, and low expression of GPR12 is associated with unfavorable patient survival. Because GPR12 has been confirmed in vitro to mediate tumor cell apoptosis and migration, we postulate that GPR12 could be a novel target in pathogenesis and progression of EC and HC.

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## DISCLOSURE

The authors have no conflicts of interest to report.

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