

HTR7 Promotes Laryngeal Cancer Growth Through Activating PI3K/AKT Pathway

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Research

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Abstract

Background: Laryngeal cancer is a common malignancy of the head and neck, G protein-coupled receptors (GPCRs) are easily druggable in diseases. The 5-hydroxytryptamine receptor 7 (HTR7) belongs to the GPCR family; however, its role in laryngeal cancer remains unknown.

Methods: MTT, Colony formation assay, BrdU incorporation assay, soft agar growth assay and xenograft tumor in nude mice were used to analyze the effect of HTR7 expression level on laryngeal cancer proliferation and growth.

Results: We found that HTR7 was significantly upregulated in laryngeal cancer tissues and cells, and patients with high HTR7 expression had shorter survival time than those with low HTR7 expression. Univariate and multivariate Cox regression models showed HTR7 was an independent predictive factor for the prognosis of patients with laryngeal cancer. Cell proliferation assays and an animal model showed that HTR7 overexpression promoted laryngeal cancer proliferation and growth, while HTR7 knockdown inhibited laryngeal cancer proliferation and growth. Further analysis showed HTR7 activated the PI3K/AKT pathway, characterized by increased phosphorylation of AKT, luciferase reporter activity of FOXO factors and target expression. Inhibition of the PI3K/AKT pathway in HTR7-overexpressing cells suppressed proliferation and growth, suggesting HTR7 promoted laryngeal cancer proliferation and growth by activating the PI3K/AKT pathway.

Conclusions: In summary, HTR7 is not only a target for laryngeal cancer therapy, but also a prognostic factor for the prognosis of patients with laryngeal cancer.

Background

Laryngeal cancer is a common malignancy of the head and neck, with approximately 11,000 to 13,000 cases of laryngeal cancer being diagnosed annually [1]. In the past decade, laryngeal cancer treatment has been improved through surgery, radiation, and systemic therapy, and novel agents for metastasis [2]; however, the death rate is still high. Therefore, to improve laryngeal cancer therapy, a better understanding of the regulatory mechanism of laryngeal cancer is urgently required.

The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) pathway is frequently mutated in human cancers, and there are several PI3K families exist in human cells [3]. Class I_A PI3Ks, which have been proved to regulate tumor progression, are heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit. Growth factor receptor tyrosine kinases, such as EGFR, HER2, MET, and FGFR phosphorylate adaptor proteins to bind p85, which relieves the inhibition of p110 by p85. Then, the p85-p110 heterodimer binds to lipid phosphatidylinositol-4,5-bisphosphate (PIP₂) at the plasma membrane. p110 phosphorylates PIP₂ to generate phosphatidylinositol-3,4,5-triphosphate (PIP₃). PTEN and INPP4B can dephosphorylate PIP₃ to inhibit the PI3K/AKT pathway. AKT, SGK, and PDK1 bind to PIP₃ at the plasma membrane, and then PDK1 and TORC2 phosphorylate AKT to fully activate AKT.

Activated AKT can phosphorylate many proteins involved in cell survival, proliferation, growth, and protein synthesis, such as FOXO family proteins, MDM2, and p27 [4, 5].

5-hydroxytryptamine (serotonin) receptor 7 (HTR7) is a G protein-coupled receptor (GPCR) first described in 1993, and many transcription factors can bind to its promoter, such as AP2, EGR-1, and MAZ [6, 7]. HTR7 is associated with circadian rhythm, anxiety, depression, schizophrenia, nociception, and memory [8-10]. However, role of HTR7 in tumor progression has not been studied. In the present study, we aimed to investigate the role of in laryngeal cancer. The results showed that HTR7 promoted laryngeal cancer growth by activating the AKT pathway.

Materials And Methods

Cell culture and specimens

Human oral keratinocytes cell and laryngeal cancer cell TU212, FaDu, Hep-2, TU212 and TU686 were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, BI) at 37°C in a humidified incubator with 5% CO₂. All the laryngeal cancer tissues and adjacent normal laryngeal tissues were obtained from the LSCC patients who were performed surgical procedure in the Otolaryngology Head & Neck department of Guangdong Provincial People's Hospital. Eight pairs of fresh laryngeal cancer tissues and adjacent normal laryngeal tissues were obtained during the procedures and immediately frozen in liquid nitrogen. A cohort of 113 laryngeal cancer tissues were obtained, the detailed clinicopathological characteristics were showed in Supplemental Table 1. The Ethics Committee of approved all collections. All patients gave written informed consents.

Quantitative reverse transcription (Q-PCR)

Total RNA was isolated using FastPure Cell/Tissue Total RNA isolation Kit (Vazyme) according to the manufacturer's protocols. RNA was reversely transcribed into cDNA according to the instructions of HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme). Q-PCR was performed on the CFX-96 PCR system (Bio-Rad) using AceQ Universal SYBR qPCR Master Mix (Vazyme). The relative quantification of mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ method. GAPDH was used as the negative control for detecting mRNA expression.

Western blot

Total protein was extracted from cells and tissues using RIPA buffer supplemented with protease inhibitor cocktail (Roche), protein concentration was determined using BCA Kit (Thermo). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked using 5% skimmed milk at room temperature for 1h, then membranes were incubated with primary antibodies at 4°C for overnight and incubated with horseradish peroxidase-labeled secondary antibodies. Finally, the membranes were immersed in electrochemiluminescence luminescence and exposed using a

chemiluminometer. The primary antibodies were shown as following: HTR7 (ab137493, Abcam), p21 (#2947, CST), p16 (#80772, CST), cyclin D1 (#55506, CST), cyclin E (#81045, CST), AKT (#9272, CST), p-AKT (#9611, CST) and GAPDH (#5174, CST).

Small molecule compound, vector construction, siRNA synthesis and transfection

AKT inhibitor Perifosine (s1037) was purchased from Selleck and dissolved using DMSO (Sigma). siAKT (siG150820105010-1-5) was purchased from RiBoBio. To overexpression HTR7, the CDS sequence was cloned into lentivirus vector pSin-EF2-Puro, to knockdown HTR7, two shRNA sequences were cloned into lentivirus vector PLKO.1-pur, the shRNA sequences were: shHTR7#1: 5' GCACACCAACAGAACTGAGTT' and shHTR7#2: 5' CCAGGACTTTGGCTATACGAT3'. The vectors were co-transfected with packing vectors pSPAX2 and pM2.G into 293FT using Lipofectamine 3000 (Thermo) to generate lentivirus. The lentivirus were infected cells for overnight, stable cell lines were screened using Puromycin (Sigma).

MTT

Indicated cells were seeded in a 96-well plate at a density of 4×10^3 cells/well, Six replications were prepared for treatment group. Cells were incubated with MTT solution at indicated time points for 4h at 37°C, the DMSO was added to each well. The plates were gently shaken on a horizontal shaker for 10min to dissolve crystals, the optical density (OD) value of each well was read at 570nm in a microplate reader (BioTek). Each experiment was repeated 3 times.

Colony formation assay and BrdU incorporation assay

Cells were plated at 6-well plates at a density of 0.5×10^3 cells/well and cultured for 10 days. Colonies were fixed with 10% formaldehyde for 5min and then stained with 1% crystal violet for 30s. BrdU incorporation assay was performed using BrdU Kit (eBioscience).

Soft agar growth assay

Cells were resuspended in 2ml complete medium plus 0.3% agar, the agar-cell mixture was plated on the top of a bottom layer consisting 1% agar in complete medium. 10 days after, colony size was measured using an ocular micrometer and colonies larger than 0.1mm in diameter were counted. The experiment was performed three times for each cell line.

Xenograft tumor in nude mice

Nude mice (4-week age) were purchased from Model Animal Research Center of Nanjing University and randomly assigned into 4 groups with 3 mice in each group: Vector control group, HTR7 overexpression group, Scramble group, HTR7 knockdown group. The dorsal flank of each nude mouse was injected subcutaneously with 1×10^7 cells. The length (L) and width (W) of the tumor were measured with a Vernier caliper after the occurrence of the tumor. The volume of the tumor was calculated as $V = W^2 \times L \times 0.52$. Tumor size were measured weekly. All animal experiments were performed in accordance with National

Institutes of Health guidelines and approved by the animal care and use committee of Guangdong Academy of Medical Sciences.

Statistical analysis

All data were processed using SPSS 21.0 statistical software (IBM). Measured data were expressed as mean \pm standard deviation. Comparisons between two groups were conducted with independent t-test. One-way analysis of variance (ANOVA) was used to compare difference in multiple groups. $P < 0.05$ was statistically significant.

Results

HTR7 is upregulated in laryngeal carcinoma tissues and cells

We found that *HTR7* was significantly upregulated in laryngeal cancer tissues compared with that in normal laryngeal tissues in data from The Cancer Genome Atlas (TCGA) database (Figure 1a). We also confirmed this result using eight pairs of laryngeal cancer tissues and their adjacent normal tissues: Q-PCR and western blotting showed that *HTR7* was also upregulated in laryngeal tissues (Figure 1b and 1c). Q-PCR and western blotting further demonstrated that *HTR7* was upregulated in laryngeal cells compared with that in normal laryngeal epithelia cells (Figure 1d). Gene Set Enrichment Analysis (GSEA) showed the high *HTR7* expression correlated positively with strong tumorigenesis ability (Figure 1e). Finally, further analysis of TCGA data revealed that patients with high *HTR7* expression had a shorter survival time than those with low *HTR7* expression (Figure 1f). Taken together, these findings suggested *HTR7* was upregulated in laryngeal cancer tissues and cells, and high *HTR7* expression was associated with poor outcome.

HTR7 is an independent predictive factor for prognosis of patients with laryngeal cancer

According to above results, we determined the association between *HTR7* expression and overall survival of patients with laryngeal cancer using long-rank test and multivariable Cox proportional hazard regression analysis. IHC was used to determine *HTR7* expression in cohort of 113 patients with laryngeal cancer (Figure 2a). Kaplan–Meier survival analysis showed that patients with low *HTR7* expression had longer survival than those with high *HTR7* expression (Figure 2b). To identify independent prognostic factors for the survival of patients with laryngeal cancer, univariate and multivariate Cox regression models were used, which showed that relapse, distant metastasis, and *HTR7* expression were significant prognostic predictors for overall survival of patients with laryngeal cancer. Relapse and *HTR7* expression were identified as independent predictive factors for the prognosis of patients with laryngeal cancer (Figure 2c). Taken together, these results showed high *HTR7* expression was a significant independent prognostic factor for poor prognosis in laryngeal cancer and could be used as a biomarker for prognosis in patients with laryngeal cancer.

HTR7 promotes laryngeal cancer proliferation *in vitro*

To determine the function of HTR7 in laryngeal cancer progression, we used GSEA to analysis the relationship between *HTR7* expression and the expression of key genes associated with tumor proliferation and found that *HTR7* expression correlated positively with *CCND1* expression (Figure 3a). *CCND1* is a key factor for G1/S transition, and accelerates cell cycle progression [11, 12]. Cyclin E promotes the cell cycle, and p21 and p16 inhibit the cell cycle [13-15]. We overexpressed *HTR7* in the FaDu cell line, which has low *HTR7* expression, and knocked down *HTR7* expression in TU212, which has high *HTR7* expression. Western blotting analysis showed *HTR7* overexpression increased cyclin D1 and cyclin E levels and reduced p16 and p21 levels (Figure 3b), while *HTR7* knockdown reduced cyclin D and cyclin E levels and increased p21 and p16 levels (Figure 4a). The MTT assay showed that *HTR7* overexpression promoted cell proliferation significantly (Figure 3c), while *HTR7* knockdown inhibited cell proliferation significantly (Figure 4b). Colony formation assays showed that *HTR7* overexpression increased cell proliferation (Figure 3d), while *HTR7* knockdown inhibited cell proliferation (Figure 4c). BrdU incorporation assays revealed that *HTR7* overexpression promoted cell proliferation (Figure 3e), while *HTR7* knockdown inhibited cell proliferation (Figure 4d). These results suggested that HTR7 promoted the proliferation of laryngeal cancer.

HTR7 promotes laryngeal cancer growth *in vivo*

To confirm above results, we used soft agar growth assays and a mouse model to determine the role of HTR7 in laryngeal cancer growth. Soft agar growth assays demonstrated that *HTR7* overexpression promoted tumor anchorage-independent growth significantly, while *HTR7* knockdown inhibited tumor anchorage-independent growth significantly (Figure 5a). The mouse model revealed that *HTR7* overexpression increased the tumor volume significantly, while *HTR7* knockdown reduced the tumor volume significantly (Figure 5b). These results suggested that HTR7 promoted laryngeal cancer growth.

HTR7 promotes laryngeal cancer growth by activating the PI3K/AKT pathway

To determine the regulatory mechanism of HTR7 in laryngeal cancer growth, we used GSEA to analyze the signaling pathways regulated by HTR7 and found that *HTR7* expression levels correlated positively with those of PI3K/AKT pathway targets (Figure 6a). Luciferase reporter assays showed that *HTR7* overexpression significantly increased the luciferase activity, while *HTR7* knockdown significantly inhibited the luciferase activity, suggesting that HTR7 increased PI3K/AKT pathway activity (Figure 6b). Western blotting assays showed that HTR7 overexpression increased the level of phosphorylated AKT, while *HTR7* knockdown reduced the level of phosphorylated AKT (Figure 6c), suggesting that HTR7 activated the AKT pathway. BCL2L1, BCL2A1, BIRC5, BCL2, XIAP, CCNE2, CCND2, CDK2, CDK4, and BAD are targets of AKT, and are associated with tumor proliferation and inhibition of apoptosis [16, 17]. Q-PCR analysis showed that HTR7 overexpression promoted their expression, while *HTR7* knockdown inhibited their expression (Figure 6d), which supported the view that HTR7 activated the PI3K/AKT pathway.

To understand whether HTR7 promoted laryngeal cancer growth by activating the AKT pathway, we inhibited PI3K/AKT pathway activation using either small interfering RNAs targeting *AKT* or the AKT pathway inhibitor perifosine [18] in *HTR7*-overexpressing laryngeal cancer cells. Colony formation assays

showed that inhibition of the PI3K/AKT pathway significantly inhibited cell proliferation (Figure 7a), which was confirmed using soft agar growth assays (Figure 7b). These results suggested HTR7 promoted laryngeal cancer growth by activating the PI3K/AKT pathway.

We confirmed these results using clinical samples. Western blotting assays showed that laryngeal cancer tissues with high HTR7 expression had high p-AKT levels, while laryngeal cancer tissues with low HTR7 expression had low p-AKT levels, and the expression of HTR7 and p-AKT levels correlated positively (Figure 7c), suggesting that HTR7 activated the PI3K/AKT pathway in clinical samples.

Discussion

In present study, we found HTR7 was significantly upregulated in laryngeal cancer cells and tissues, and patients with high HTR7 expression had a shorter survival time. HTR7 was an independent predictive factor for the prognosis of patients with laryngeal squamous cell cancer. Functional assays showed that *HTR7* overexpression promoted laryngeal cancer proliferation and growth. Mechanism analysis showed HTR7 promoted laryngeal cancer proliferation and growth by activating the AKT pathway.

Previously, the PI3K/AKT pathway has been associated with laryngeal cancer, and the phosphorylation of AKT is a marker of PI3K/AKT activation. AKT phosphorylation is associated with treatment failure of head and neck cancer, and inhibition of AKT increased radiosensitivity [19]. Many genes and non-coding RNAs have been showed to regulate laryngeal cancer progression by regulating the PI3K/AKT pathway. For example, the microRNA miR-132 promoted laryngeal cancer proliferation and growth by targeting *FOXO1*, which activated the PI3K/AKT pathway [20]. TRA2 β is associated with poor differentiation, lymph node metastasis, and advanced clinical stage of laryngeal cancer, in which it promotes proliferation, growth, invasion, and migration, and inhibits apoptosis by activating PI3K/AKT [21]. Our findings also suggested HTR7 promoted laryngeal cancer proliferation and growth through the PI3K/AKT pathway. *HTR7* overexpression increased the level of phosphorylated AKT, while *HTR7* knockdown reduced the level of phosphorylated AKT. Luciferase reporter analyses suggested that HTR7 overexpression increased FOXO transcription factor-mediated transcription. The expression of PI3K/AKT pathway target gene also increased, further demonstrating that HTR7 activated the PI3K/AKT pathway. Inhibition of PI3K/AKT pathway in *HTR7*-overexpressing cells inhibited cell proliferation and growth, suggesting that HTR7 promoted laryngeal cancer proliferation and growth by activating the PI3K/AKT pathway.

The role of HTR7 has not been studied in tumor progression until now. HTR7 belongs to the GPCR family, which is one of the most studied pharmacological targets because of their diverse biological functions and druggable sites. GPCRs represent about 34% of drugs approved by FDA, and about 27% in terms of sales [22-24], suggesting that HTR7 might be easily druggable to treat laryngeal cancer. Moreover, CAR-T or CAR-NK has been used for tumor therapy, the membrane markers of tumors are important for CAR-T or CAR-NK, HTR7 might be a well marker for laryngeal cancer treatment. we will determine whether HTR7 would be a target for CAR-T or CAR-NK therapy. In summary, HTR7 promoted laryngeal cancer growth by activating the PI3K/AKT pathway, providing a new target for laryngeal cancer prognosis and therapy.

Conclusions

To sum up, our results clearly emphasize the role of a G protein-coupled receptor HTR7 in laryngeal cancer. Patients with high HTR7 expression had shorter survival time than those with low HTR7 expression and was an independent predictive factor for patients with laryngeal cancer. HTR7 overexpression promoted laryngeal cancer proliferation and growth, it promoted laryngeal cancer proliferation and growth by activating the PI3K/AKT pathway. HTR7 is not only a target for laryngeal cancer therapy, but also a prognostic factor for the prognosis of patients with laryngeal cancer.

Abbreviations

HTR7: 5-hydroxytryptamine receptor 7; GPCRs: G protein-coupled receptors; GSEA: Gene Set Enrichment Analysis; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT: protein kinase B; PIP2: lipid phosphatidylinositol-4,5-bisphosphate; PIP3: phosphatidylinositol-3,4,5-triphosphate.

Declarations

Ethics approval and consent to participate

For human samples, the Ethics Committee of approved all collections. All patients gave written informed consents. All animal experiments were performed in accordance with National Institutes of Health guidelines and approved by the animal care and use committee of Guangdong Academy of Medical Sciences.

Consent for publication

Not applicable

Availability of data and materials

All data are available in the manuscript or upon request to the authors.

Competing Interests

None

Funding

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Not applicable

Author contributions

SYZ and TL designed the study. XLS, WLL, ZML, MMX and RL performed experiments and analyzed data. YXL generated stable cell lines. YXL collected clinical samples. SYZ and TL supervised the study. All authors edited the manuscript.

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Figures

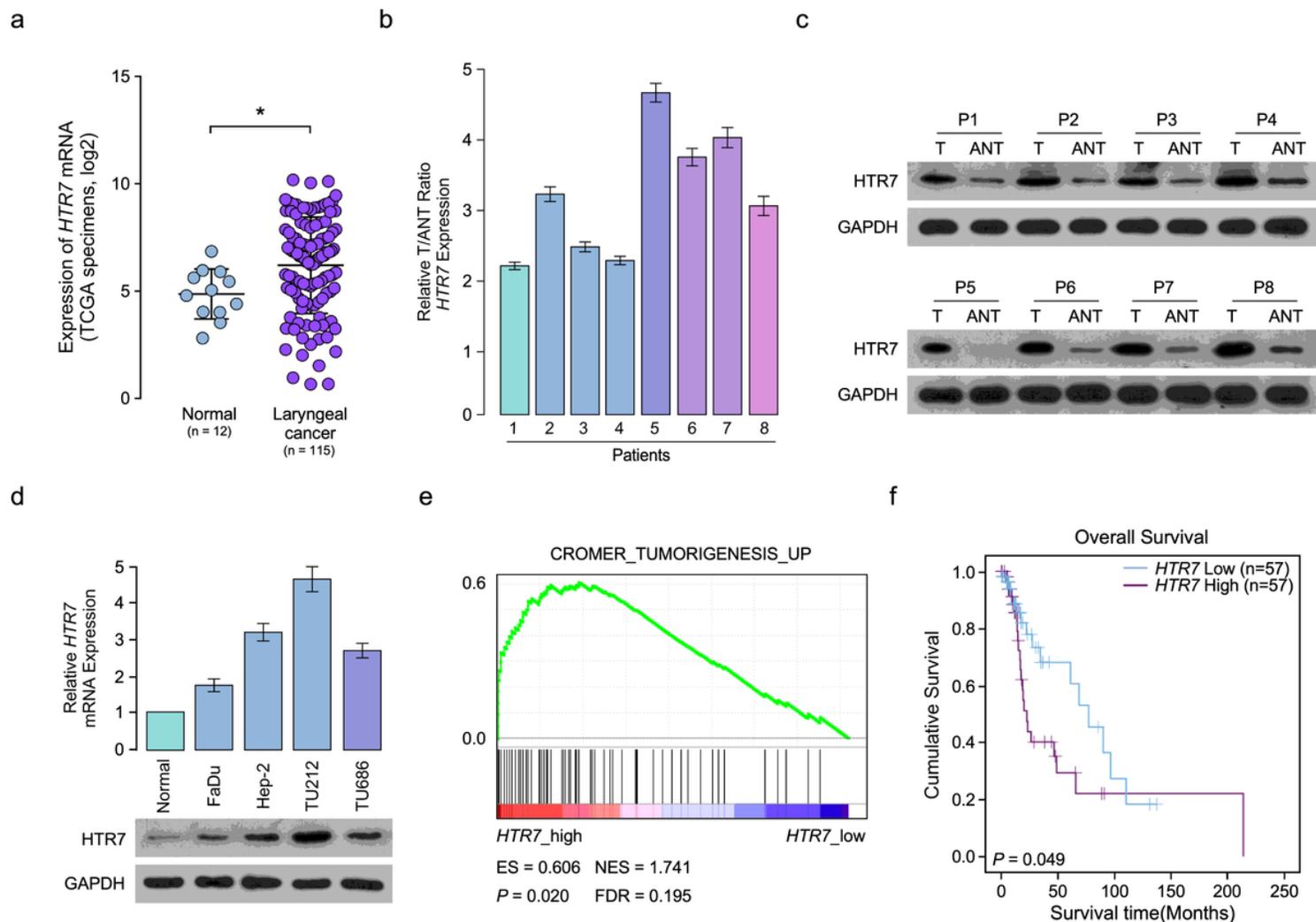
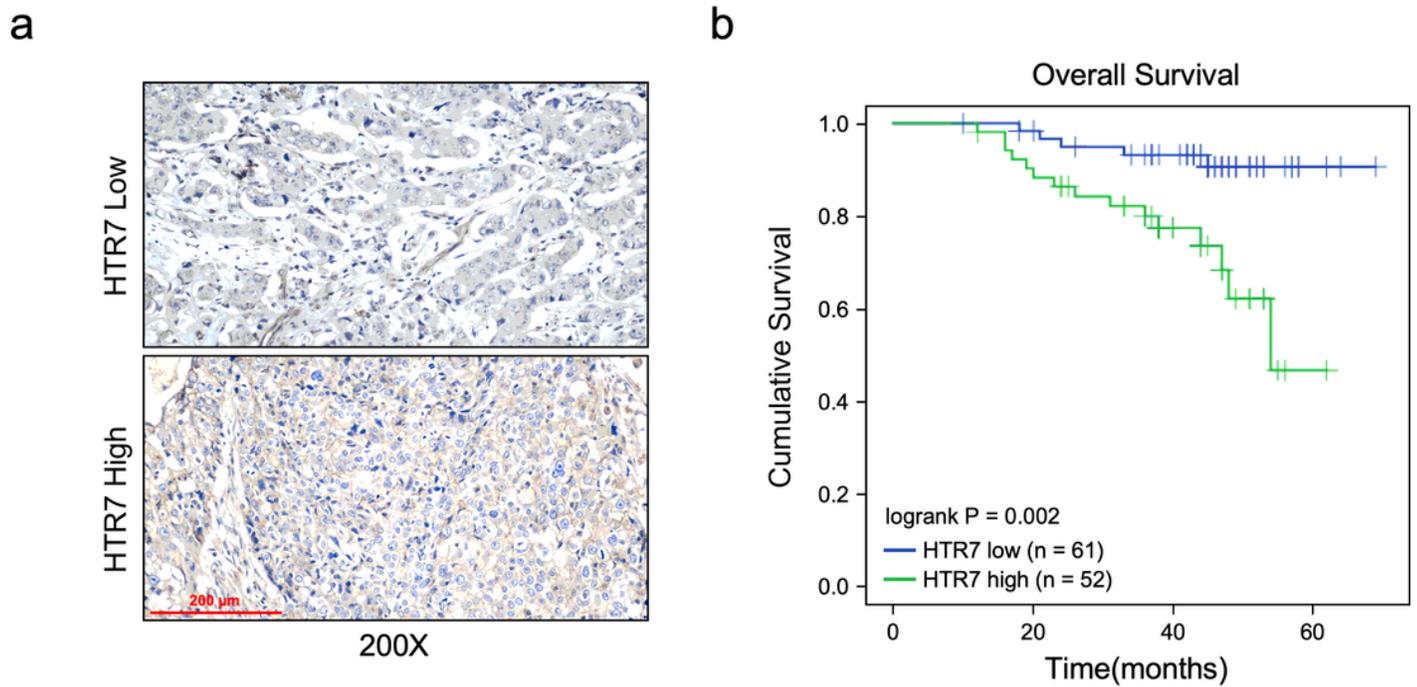


Figure 1

HTR7 is overexpressed in laryngeal cancer tissues and cells. a. HTR7 was significantly upregulated in laryngeal cancer tissues compared with that in normal laryngeal tissues; data were downloaded from the TCGA. b. QPCR analysis of HTR7 expression in laryngeal cancer tissues (T) and adjacent normal tissues (ANT). c. Western blotting analysis of HTR7 levels in laryngeal cancer tissues (T) and adjacent normal tissues (ANT). GAPDH was used as the loading control. d. QPCR and western blotting analysis of HTR7 expression in laryngeal cancer cells and normal laryngeal epithelial cells. GAPDH was used as the loading control for western blot analysis. e. GSEA analysis of the relationship between HTR7 expression levels and tumorigenesis. f. Survival curve analysis of HTR7 expression and clinical outcome. Error bars represent the SEM. * $P < 0.05$.



c

Univariate and multivariate analyses of various prognostic parameters
in patients with laryngeal cancer by Cox-regression analysis

	Univariate analysis			Multivariate analysis		
	No. patients	P	Relative risk	P	Relative risk	95% confidence interval
Relapse						
Yes	35	0.005	3.596	0.017	2.966	1.214 - 7.248
No	78					
Distant metastasis						
Yes	10	0.002	5.081	0.068	2.732	0.930 - 8.026
No	103					
Expression of HTR7						
Low expression	61	0.004	4.496	0.030	3.262	1.118 - 9.517
High expression	52					

Figure 2

HTR7 is an independent predictive factor for prognosis of patients with laryngeal cancer. a. Representational IHC figure for low and high HTR7 expression. b. Overall survival analysis for HTR7 expression and patient survival time. c. Univariate and multivariate analyses of relapse, distant metastasis, and HTR7 expression in patients with laryngeal cancer using Cox regression analysis. Error bars represent the SEM. * $P < 0.05$.

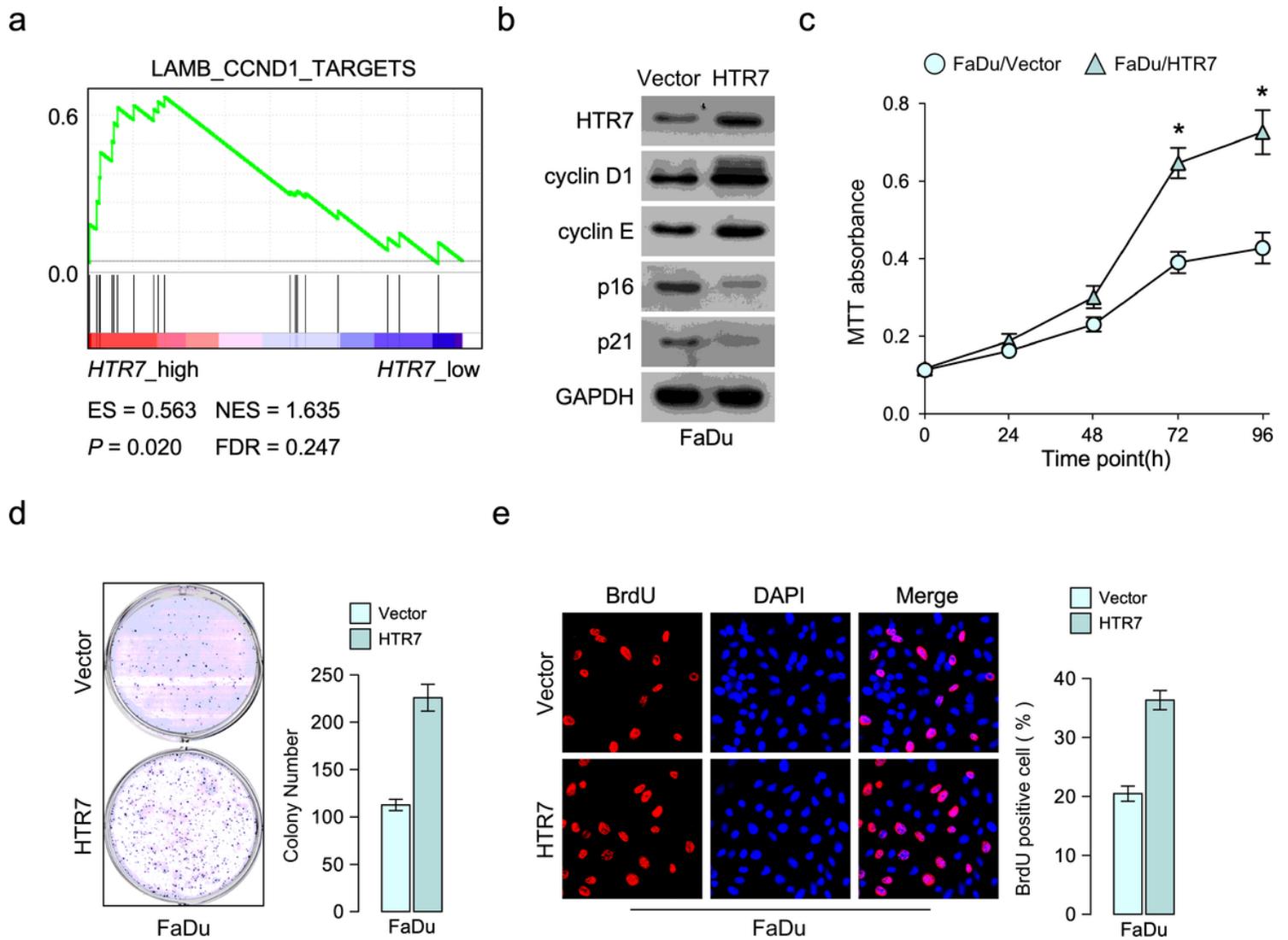


Figure 3

HTR7 overexpression promotes laryngeal cancer proliferation. a. GSEA analysis of the relationship between HTR7 expression and CCND1 expression. b. western blotting analysis of cyclin D1, cyclin E, p16, and p21 levels when HTR7 was overexpressed in FaDu cells. GAPDH was used as the loading control. c. MTT assay of the effect of HTR7 overexpression on laryngeal cancer proliferation. d. Colony formation assay of the effect of HTR7 overexpression on laryngeal cancer proliferation. e. BrdU incorporation assay of the effect of HTR7 overexpression on laryngeal cancer proliferation. Error bars represent the SEM. *P < 0.05.

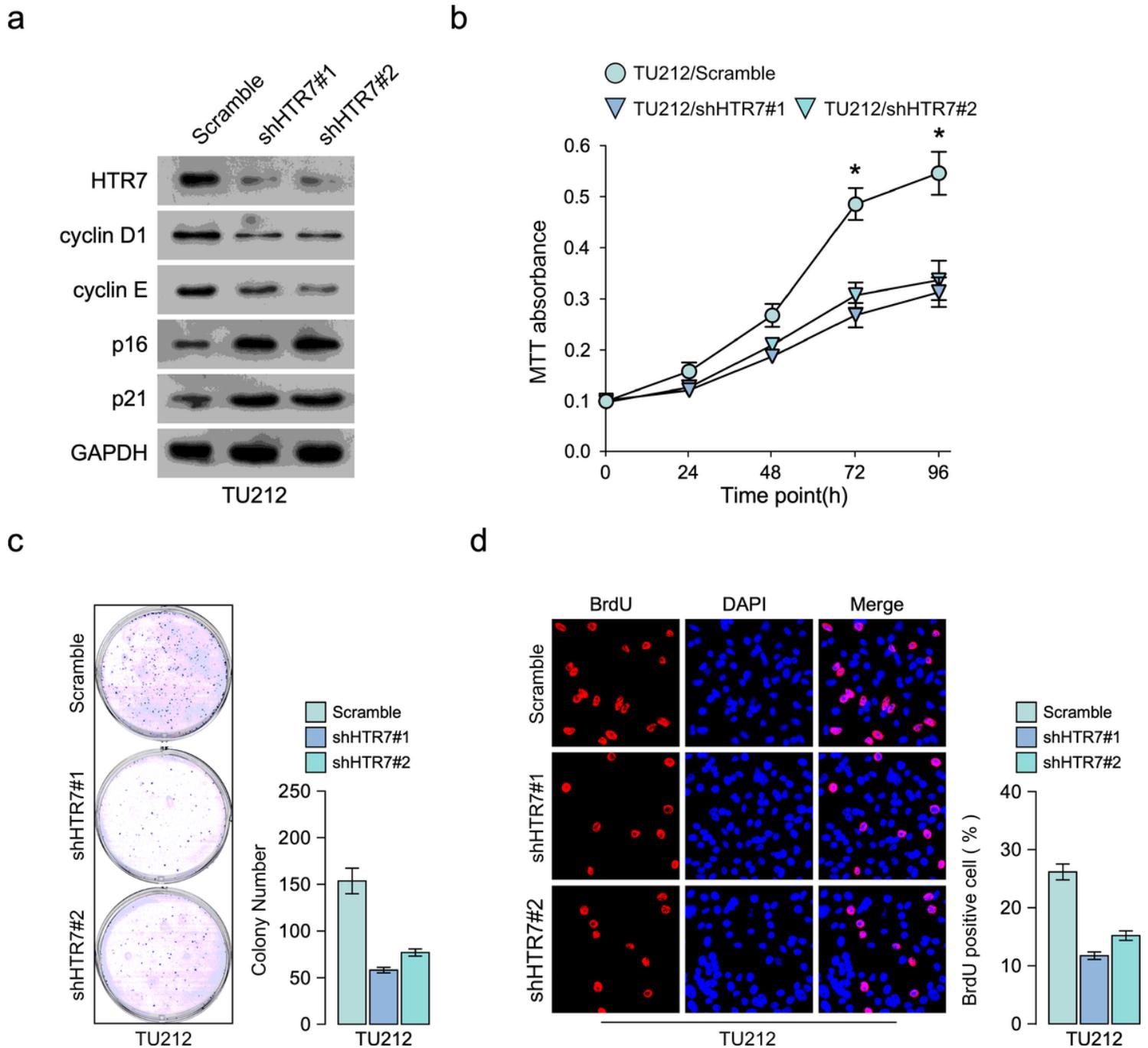


Figure 4

HTR7 knockdown inhibits laryngeal cancer proliferation. a. Western blotting analysis of cyclin D1, cyclin E, p16, and p21 levels when HTR7 was knocked down in TU212 cells. GAPDH was used as the loading control. b. MTT assay of the effect of HTR7 knockdown on laryngeal cancer proliferation. d. Colony formation assay of the effect of HTR7 knockdown on laryngeal cancer proliferation. e. BrdU incorporation assay of the effect of HTR7 knockdown on laryngeal cancer proliferation. Error bars represent the SEM. *P < 0.05.

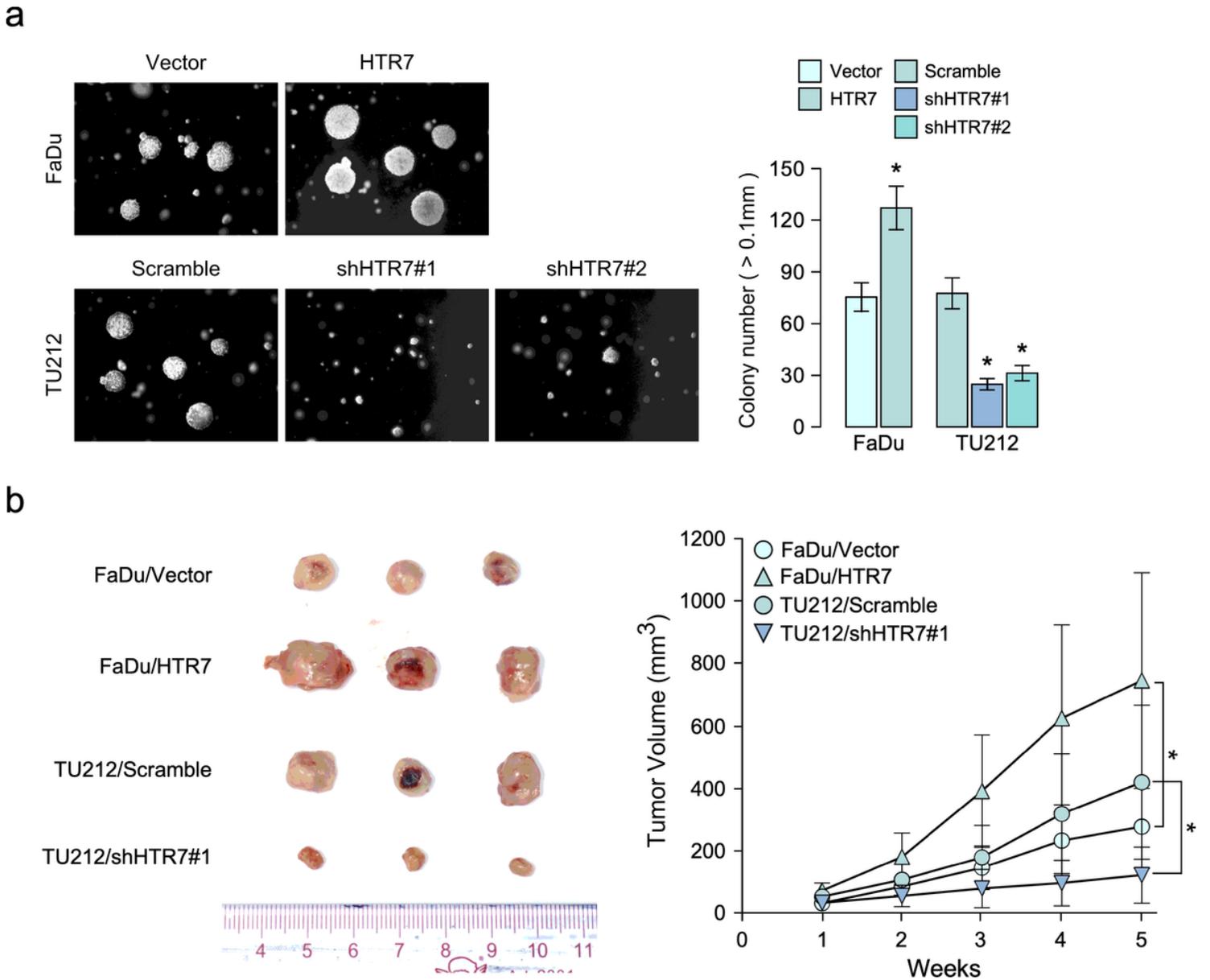


Figure 5

HTR7 promotes laryngeal cancer growth. a. Soft agar growth analysis of the effect of HTR7 overexpression or knockdown on cell growth. Representational figures for soft agar growth assay are shown on the left, the statistical analysis is shown on the right. b. Animal model analysis of the effect of HTR7 overexpression or knockdown on cell growth. Representational figures for soft agar growth assay are shown on the left, the tumor volume analysis is shown on the right. Error bars represent the SEM. *P < 0.05.

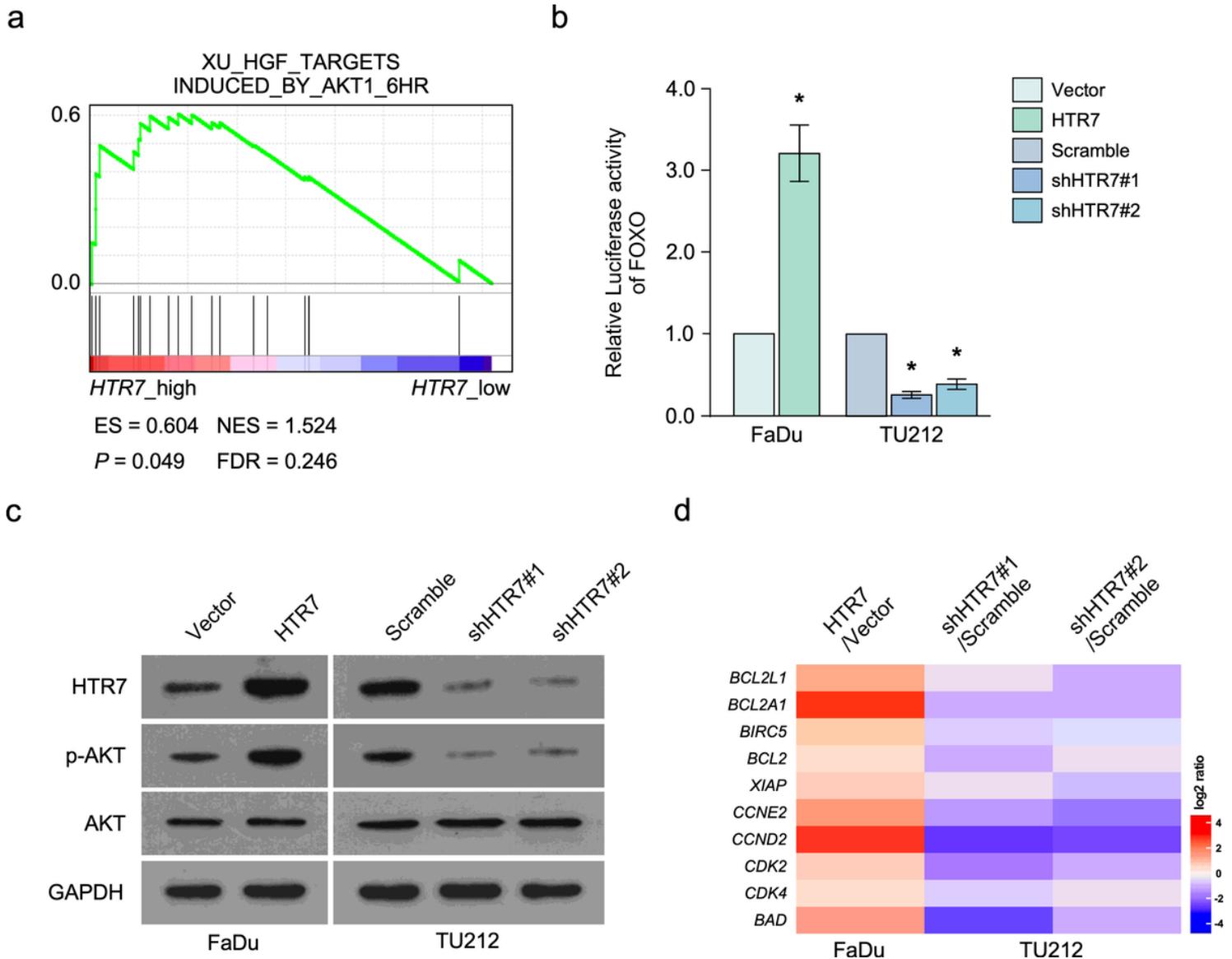


Figure 6

HTR7 activated the AKT pathway. a. GSEA analysis of the correlation between HTR7 expression and the expression of AKT pathway target genes. b. Luciferase reporter analysis of FOXO transcription activity after HTR7 overexpression or knockdown. c. Western blotting analysis of AKT, p-AKT, and HTR7 levels after HTR7 overexpression or knockdown, GAPDH was used as the loading control. d. QPCR analysis of BCL2L1, BCL2A1, BIRC5, BCL2, XIAP, CCNE2, CCND2, CDK2, CDK4, and BAD expression after HTR7 overexpression or knockdown, the results are shown using a heatmap. Error bars represent the SEM. *P < 0.05.

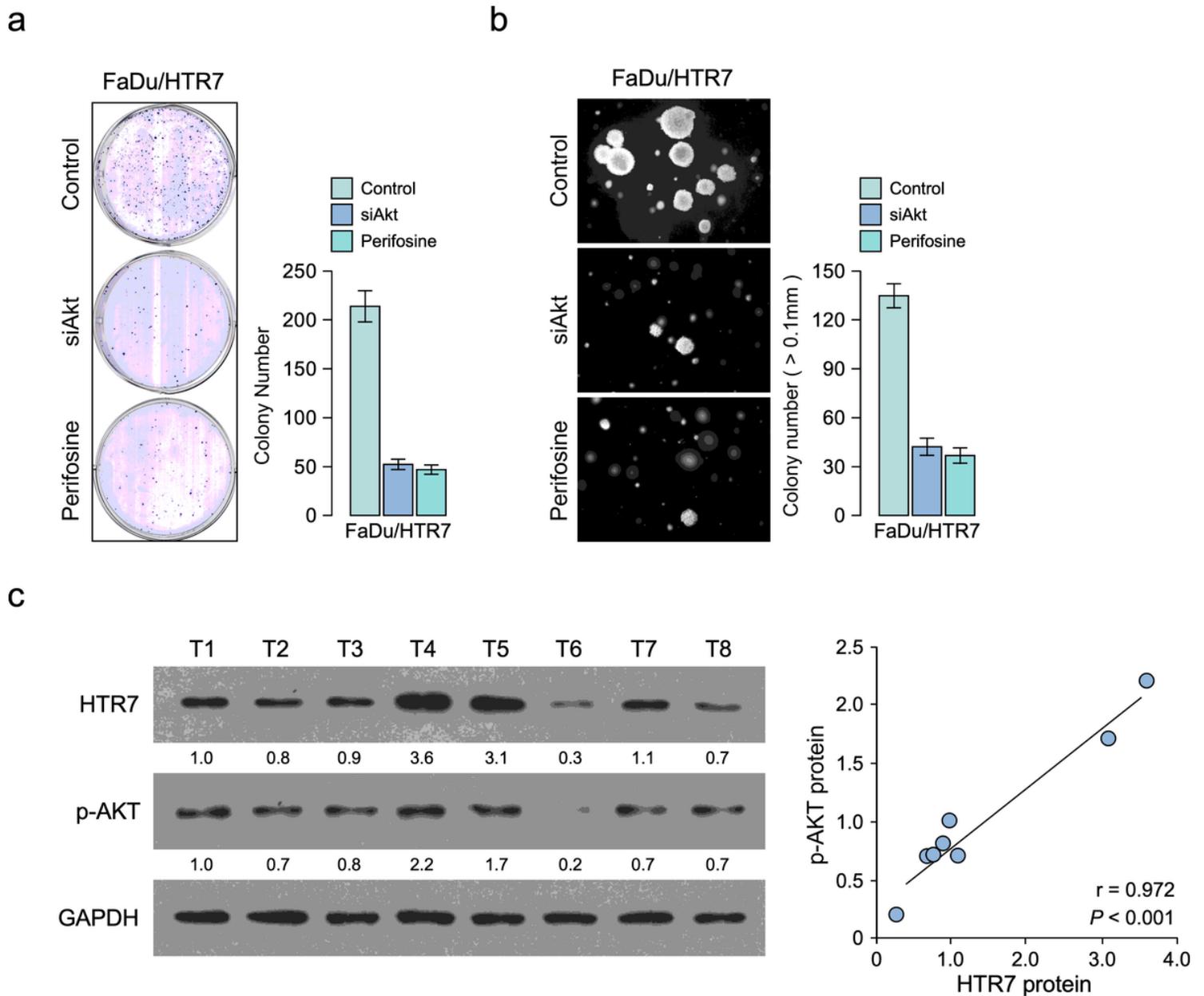


Figure 7

HTR7 promotes laryngeal cancer growth by activating the AKT pathway. a. Colony formation assay of the effect on cell growth of inhibition of the AKT pathway in HTR7-overexpressing cells. b. Soft agar growth analysis of the effect on cell growth of inhibition of the AKT pathway in HTR7-overexpressing cells. Representative images for the soft agar growth assay are showed on the left, the statistical analysis is shown on the right. c. Western blotting analysis of HTR7 and p-AKT levels in laryngeal cancer tissues, and the statistical analysis of the relationship between HTR7 and p-AKT levels. GAPDH was used as the loading control. Error bars represent the SEM. * $P < 0.05$.

Supplementary Files

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- [SupplementalTable1.docx](#)