HTR7 promotes laryngeal cancer growth through PI3K/AKT pathway activation

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

Methods: Colony formation assay, Soft agar growth assay, BrdU incorporation assay and MTT assay were used to analyze the effect of HTR7 on laryngeal cancer cell proliferation. Xenograft tumors in nude mice was used to analyze the effect of HTR7 on laryngeal cancer growth. Luciferase reporter assay was used to analyze the effect of HTR7 on phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) pathway activity.

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 that 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 forkhead box O (FOXO) factors, and target expression. Inhibition of the PI3K/AKT pathway in HTR7-overexpressing cells suppressed proliferation and growth, suggesting that HTR7 promotes laryngeal cancer proliferation and growth by activating the PI3K/AKT pathway.

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

Keywords: 5-hydroxytryptamine receptor 7; laryngeal cancer; protein kinase B (AKT); tumor growth

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Introduction

Laryngeal cancer is a common malignancy of the head and neck, with approximately 11,000–13,000 cases of laryngeal cancer being diagnosed annually in China (1). In the past decade, laryngeal cancer treatment has improved with surgery, radiation, 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 several PI3K families exist in human cells (3). Class IA PI3Ks, which are known 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. The p85-p110 heterodimer then 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, the role of HTR7 in tumor progression has not been studied. In the present study, we aimed to investigate the role of HTR7 in laryngeal cancer. The findings indicated that HTR7 promotes laryngeal cancer growth by activating the AKT pathway. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-21-1069).

Methods

Cell culture and specimens

Human oral keratinocytes cell and laryngeal cancer cell lines, including Tu212, FaDu, Hep-2, Tu212, and Tu686 were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Beit-Haemek, Israel) at 37 °C in a humidified incubator with 5% CO2. All laryngeal cancer tissues and adjacent normal laryngeal tissues were obtained from LSCC patients who underwent surgery at the Otolaryngology Head and Neck Department of Guangdong Provincial People’s Hospital (Guangzhou, Guangdong, China). Eight pairs of fresh laryngeal cancer tissues and adjacent normal laryngeal tissues were obtained during the procedures and immediately frozen in liquid nitrogen. A total of 113 laryngeal cancer tissues were obtained; the detailed clinicopathological characteristics are shown in Table S1. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The Ethics Committee of Guangdong Provincial People’s Hospital and Guangdong Academy of Medical Sciences approved all collections. All patients provided written informed consent.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the FastPure Cell/Tissue Total RNA isolation Kit (Vazyme, Nanjing, Jiangsu, China) according to the manufacturer’s protocols. RNA was reversely transcribed into cDNA according to the instructions of the HiScript III 1st Strand cDNA Synthesis Kit (+ gDNA wiper; Vazyme). qRT-PCR was performed on the CFX-96 PCR system (Bio-Rad) using the AceQ Universal SYBR qPCR Master Mix (Vazyme). The relative quantification of mRNA expression was calculated using the 2^−ΔΔCt method. GAPDH was used as the negative control for detecting mRNA expression.

Western blotting

Total protein was extracted from cells and tissues using RIPA buffer supplemented with protease inhibitor cocktail (Sigma, St. Louis, MI, USA), protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked using 5% skimmed milk at room temperature for 1 h. The membranes were then incubated with primary antibodies at
4°C 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 as follows: HTR7 (ab137493; Abcam, Cambridge, UK), p21 (#2947, CST, Danvers, MA, USA), 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 (siG15082010501-1-5) was purchased from RiBoBio (Guangzhou, Guangdong, China). To overexpress HTR7, the CDS sequence was cloned into lentivirus vector pSin-EF2-Puro; to knockdown HTR7, 2 shRNA sequences were cloned into lentivirus vector PLKO.1-pur. The shRNA sequences were: shHTR7#1: 5’-GCACACCAAGAATCTGTT3’ and shTHR7#2: 5’-CCAGGACTTTGGCTATACGAT-3’. The vectors were co-transfected with packing vectors pSPAX2 and pM2. G into 293FT using Lipofectamine 3000 (Thermo Fisher Scientific, USA) to generate lentivirus. The lentivirus were infected cells for overnight; stable cell lines were screened using Puromycin (Sigma).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Indicated cells were seeded in a 96-well plate at a density of 4x10^4 cells/well, six replications were prepared for the treatment group. Cells were incubated with MTT solution at the indicated time points for 4 h at 37 °C; DMSO was added to each well. The plates were gently shaken on a horizontal shaker for 10 min to dissolve crystals; the optical density value of each well was read at 570 nm in a microplate reader (BioTek). Each experiment was repeated 3 times.

Colony formation assay and BrdU incorporation assay

Cells were seeded in 6-well plates at a density of 0.5x10^5 cells/well and cultured for 10 days. Colonies were fixed with 10% formaldehyde for 5 min and then stained with 1% crystal violet for 30 s. BrdU incorporation assay was performed using the BrdU Kit (eBioscience, San Diego, CA, USA).

Soft agar growth assay

Cells were resuspended in 2 mL complete medium plus 0.3% agar. The agar–cell mixture was plated on the top of a bottom layer consisting of 1% agar in complete medium. Ten days later, colony size was measured using an ocular micrometer, and colonies >0.1 mm in diameter were counted. The experiment was performed 3 times for each cell line.

Xenograft tumors in nude mice

All animal experiments were performed in accordance with the guidelines for animal care and under the protocols approved by the Institutional Animal Care and Use Committee of the Guangdong Provincial People’s Hospital and Guangdong Academy of Medical Sciences. Nude mice (4 weeks old) were purchased from the Model Animal Research Center of Nanjing University and randomly assigned into the following 4 groups, with 3 mice in each group: the vector control group, the HTR7 overexpression group, the scramble group, and the HTR7 knockdown group. The dorsal flank of each nude mouse was injected subcutaneously with 1x10^7 cells. The length (L) and width (W) of the tumors were measured with a Vernier caliper. The volume of the tumor was calculated as Volume (V) = W^2 × L × 0.52. Tumor size were measured weekly.

Statistical analysis

All data were processed using SPSS version 21.0 (IBM, Armonk, NY, USA). Measured data were expressed as mean ± standard deviation. Comparisons between 2 groups were conducted with independent t-test. One-way analysis of variance was used to compare difference in multiple groups. P<0.05 indicated statistical significance.

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.
Sheng et al. HTR7-PI3K-AKT axis promotes laryngeal cancer

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Figure 1 5-hydroxytryptamine receptor 7 (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 Cancer Genome Atlas. (B) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of HTR7 expression in laryngeal cancer tissues (T) and adjacent normal tissues (ANT). (C) Western blot analysis of HTR7 levels in laryngeal cancer tissues (T) and adjacent normal tissues (ANT). GAPDH was used as the loading control. (D) qRT-PCR and Western blot analysis of HTR7 expression in laryngeal cancer cells and normal laryngeal epithelial cells. GAPDH was used as the loading control for the Western blot analysis. (E) Gene Set Enrichment 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.

(TCGA) database (Figure 1A). We also confirmed this result using 8 pairs of laryngeal cancer tissues and their adjacent normal tissues: qRT-PCR and Western blotting showed that HTR7 was also upregulated in laryngeal tissues (Figure 1B,C). qRT-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 was positively correlated with strong tumorigenesis ability (Figure 1E). Finally, a 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 that 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 the prognosis of patients with laryngeal cancer**

On the basis of these results, we determined the association between HTR7 expression and the overall survival of
patients with laryngeal cancer using log-rank test and multivariable Cox proportional hazard regression analysis. Immunohistochemistry was used to determine HTR7 expression in the 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 the 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 that 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 analyze the relationship between HTR7 expression and the expression of key genes associated with tumor proliferation; HTR7 expression was...
found to be positively correlated 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 first determined the effect of HRT7 level on laryngeal cancer cells, MTT analysis showed TU212 with high HTR7 level had higher proliferation rate than FaDu with low HTR7 level (Figure S1). 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 blot analysis showed that HTR7 overexpression increased cyclin D1 and cyclin E levels and reduced p16 and p21 levels (Figure 3B). The MTT assay showed that HTR7 overexpression significantly promoted cell proliferation (Figure 3C). Colony formation assays showed that HTR7 overexpression increased cell proliferation (Figure 3D) and BrdU incorporation assays revealed that HTR7 overexpression promoted cell proliferation (Figure 3E). While HTR7 knockdown reduced cyclin D and cyclin E levels and increased p21 and p16 levels (Figure 4A), moreover cell proliferation was significantly inhibited (Figure 4B,C,D). These results suggested that HTR7 promoted the proliferation of laryngeal cancer.

**HTR7 promotes laryngeal cancer growth in vivo**

To confirm these 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 significantly promoted tumor anchorage-independent growth, while HTR7 knockdown significantly inhibited tumor anchorage-independent growth (Figure 5A). The mouse model revealed that HTR7 overexpression significantly increased the tumor volume, while HTR7 knockdown significantly reduced the tumor volume (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 were positively correlated with those of PI3K/AKT pathway targets (Figure 6A). Luciferase reporter assays showed that HTR7 overexpression significantly increased luciferase activity, while HTR7 knockdown significantly inhibited luciferase activity, suggesting that HTR7 increased PI3K/AKT pathway activity (Figure 6B). Western blot 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. BCL2 like 1 (BCL2L1), BCL2 related protein A1 (BCL2A1), Baculoviral IAP repeat containing 5 (BIRC5), BCL2 apoptosis regulator (BCL2), X-linked inhibitor of apoptosis (XIAP), cyclin E2 (CCNE2), cyclin D2 (CCND2), cyclin dependent kinase 2 (CDK2), cyclin dependent kinase...
4 (CDK4), and BCL2 associated agonist of cell death (BAD) are targets of AKT and are associated with tumor proliferation and the inhibition of apoptosis (16,17). qRT-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, in HTR7-overexpressing laryngeal cancer cells (18). Colony formation assays showed that the inhibition of the PI3K/AKT pathway significantly inhibited cell proliferation (Figure 7A), which was confirmed using soft agar growth assays (Figure 7B). These findings indicated that HTR7 promoted laryngeal cancer growth by activating the PI3K/AKT pathway.

We confirmed these results using clinical samples. Western blot 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 were positively correlated (Figure 7C), suggesting that HTR7 activated the PI3K/AKT pathway in clinical samples.

**Discussion**

In the present study, we found that 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 that HTR7 promoted laryngeal cancer proliferation and growth by activating the AKT pathway.

The PI3K/AKT pathway has been previously found to be 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 the inhibition of AKT increases radiosensitivity (19). Many genes and non-coding RNAs have been shown to regulate laryngeal cancer progression by regulating the PI3K/AKT pathway. For example, miR-132 promotes laryngeal cancer proliferation and growth by targeting FOXO1, which activates the PI3K/AKT pathway (20). TRA2\beta is associated with poor differentiation, lymph node metastasis, and advanced clinical stage of laryngeal cancer. It promotes proliferation, growth, invasion, and migration, and inhibits apoptosis by activating PI3K/AKT (21). Our findings also indicated that 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 the PI3K/AKT pathway target gene also increased, further demonstrating that HTR7 activated the PI3K/AKT pathway.

Figure 6 5-hydroxytryptamine receptor 7 (HTR7) activated the protein kinase B (AKT) pathway. (A) Gene Set Enrichment 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 blot analysis of AKT, p-AKT, and HTR7 levels after HTR7 overexpression or knockdown; GAPDH was used as the loading control. (D) Quantitative reverse transcription polymerase chain reaction analysis of BCL2L1, BCL2A1, BIRC5, BCL2, XIAP, CCNE2, CCND2, CDK2, CDK4, and BAD expression after HTR7 overexpression or knockdown; results are shown using a heatmap. Error bars represent the mean ± STDEV. *P<0.05.
AKT pathway. The inhibition of the 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.

To the best of our knowledge, the role of HTR7 in tumor progression has not been studied until now. HTR7 belongs to the GPCR family, which is one of the most studied pharmacological targets because of its diverse biological functions and druggable sites. GPCRs represent about 34% of drugs approved by the US Food and Drug Administration, and about 27% in terms of sales (22-24), suggesting that HTR7 might be easily druggable to treat laryngeal cancer. In summary, HTR7 promotes laryngeal cancer growth by activating the PI3K/AKT pathway, providing a new target for laryngeal cancer prognosis and therapy.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at [http://dx.doi.org](http://dx.doi.org).
Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-21-1069

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-21-1069). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The Ethics Committee of Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences approved all collections. All patients provided written informed consent. All animal experiments were performed in accordance with the guidelines for animal care and under the protocols approved by the Institutional Animal Care and Use Committee of the Guangdong Provincial People's Hospital and Guangdong Academy of Medical Sciences.

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References

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(English Language Editor: R. Scott)
**Table S1** Clinicopathological characteristics of studied patients and expression of 5-hydroxytryptamine receptor 7 (HTR7) in laryngeal cancer

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**Figure S1** High 5-hydroxytryptamine receptor 7 (HTR7) expression promotes laryngeal cancer proliferation. MTT analysis of laryngeal cancer cells with different HTR7 expression. *P<0.05.