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ORIGINAL RESEARCH

# RETRACTED ARTICLE: The Down-Regulation of TrkB Alleviates the Malignant Biological Behavior and Cancer Stem-Like Property of Laryngeal Cancer





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**Background:** This study aimed to evaluate the effect of TrkB down-regulation on the malignant biological behavior and stem-like characteristics of laryngeal cancer.

**Methods:** The relationship was analyzed between TrkB and clinicopathological parameters in patients with laryngeal cancer. The mRNA expression levels of TrkB and miR-10a-5p were detected by qRT-PCR in laryngeal cancer tissues and cell lines. In vitro, Hep-2 and AMC-HN-8 cell proliferation, apoptosis and stem-like properties were detected by colony formation assay, flow cytometry, sphere formation, and Western blot, respectively. In vivo, the BALB/c nude mice model was used to evaluate the effect of TrkB on tumor growth.

**Results:** The results showed that TrkB was related to smoking history, clinical stage, and lymph node metastasis, but had nothing to do with the gender, age, and tumor location of patients with laryngeal cancer. TrkB was highly expressed and miR-10a-5p was lowly expressed in laryngeal cancer tissues and cell lines. Down-regulation of TrkB inhibited Hep-2 and AMC-HN-8 cell proliferation and sphere formation as well as enhanced apoptosis. The results showed that miR-10a-5p bound to the 3′-UTR of BDNF by a dual-luciferase reporter assay. Down-regulation of miR-10a-5p induced up-regulation of TrkB promoting development of laryngeal cancer. In vivo, down-regulation of TrkB suppressed tumor growth and inhibited the expression of stem-like marker proteins and promoted apoptosis.

**Conclusion:** In conclusion, down-regulation of TrkB plays an important role in laryngeal cancer and is a promising target for future intervention strategies.

**Keywords:** TrkB, laryngeal cancer, biological behavior, cancer stem-like, apoptosis

## Introduction

Cancer incidence and mortality have been increasing in China, making cancer the leading cause of death since 2015 and a major public health problem in the country.[1] Laryngeal cancer is the eleventh most common cancer worldwide with a high mortality rate, and occurs more commonly in men than women.[2] It turns out that smoking has a linear relationship with the occurrence of laryngeal cancer, with a risk for smokers that is 10–15-times higher than the risk for nonsmokers, and the heaviest smokers have as much as a 30-times greater risk.[3] Although great advances in surgery and radiotherapy have been achieved over the past years, the prognosis for patients with advanced laryngeal cancer remains dispiriting.[4] MicroRNAs play an important role in the development and progression of cancer, where they can act as a tumor suppressor, or oncogenes.[5] The differential expression of miRNAs may be related to the early onset and development of laryngeal carcinoma.[6]

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Interestingly, Several miRNAs have been reported to be associated with perturbation of the BDNF/TrkB pathway.[7]

TrkB is a 145-kDa receptor tyrosine kinase which can be activated by brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4).[8] BDNF is best known as a neurotrophic factor that promotes survival of neurons and plays a critical role during brain development.[9,10] Recent evidence has emphasized the importance of the BDNF/TrkB signaling pathway in the regulation of carcinogenesis and metastasis.[7] BDNF triggers the TrkB/PLC gamma1 signaling pathway to promote proliferation and invasion of ovarian cancer cells through inhibition of apoptosis.[11] MiR-1-3p has significant effects on viability, proliferation, invasion, and apoptosis of bladder cancer cells by regulating the BDNF-TrkB pathway.[12] However, previous research has provided the first evidence that BDNF/TrkB signaling plays a role in resistance to anti-epidermal growth factor receptor (EGFR) blockade in treatment of colorectal cancer.[13] Importantly, our previous findings have exhibited that TrkB are overexpressed in laryngeal cancer. TrkB signaling is involved in the tumorigenicity of laryngeal cancer.[14]

Thus, this study analyzed the relationship between TrkB and gender, age, smoking history, clinical stage, lymph node metastasis, and tumor site in patients with laryngeal cancer. At the same time, TrkB plays a role in laryngeal cancer cell proliferation, apoptosis and cancer stem-like property, and the tumor growth in vivo.

## Materials and Methods

### Data Collection

A total of 69 surgically removed laryngeal cancer tissue and paracancerous tissue samples were collected from patients who received surgical resection treatment at the Affiliated Hospital of Southwest Medical University from January 2009 to December 2018. Informed consent for tissue use was provided beforehand by all patients, and the study was approved by the ethics committee of the Affiliated Hospital of Southwest Medical University. Laryngeal carcinoma was confirmed by pathological study.

### Cell Culture and Transfection

The laryngeal cancer cell lines Hep-2, TU177, TU686, and AMC-HN-8 and normal epithelial cell NP69 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Eagle's Minimum Essential Medium (EMEM, Gibco) at 37°C in a tissue culture chamber with 95% $O_2$ and 5% $CO_2$. TrkB-shRNA1, TrkB-shRNA2, and TrkB-shRNA3 were transfected into Hep2 and AMC-HN-8 cells with lipofectamine 2000 reagent (Life Technologies Corporation) according to the manufacturer's instructions. The shRNA oligo sequences are provided: TrkB-shRNA1-F: 5′TCCTAATATGTATTGGGATGTTCTCGAGAACATCCCAATACATATTAGGTTTTTC3′, TrkB-shRNA1-R: 3′TCGAGAAAAACCTAATATGTATTGGGATGTTCTCGAGAACATCCCAATACATATTAGGA5′; TrkB-shRNA2-F:5′TGCGCTTCAGTGGTTCTATAACCTCGAGGTTATAGAACCACTGAAGCGCTTTTTC3′, TrkB-shRNA2-R: 3′TCGAGAAAAAGCGCTTCAGTGGTTCTATAACCTCGAGGTTATAGAACCACTGAAGCGCA5′; TrkB-shRNA3-F: 5′TATCGTGGCATTTCCGAGATTGCTCAACAATCTCGGAAATGCCACGATTTTTC3′, TrkB-shRNA3-R: 3′TCGAGAAAAATCGTGCCATTTCGAGATTGCTCGAGCAATCTCGAAATGCCACGATA5′. To interfere with receptor tyrosine kinase signaling, cells were also treated by Trk tyrosine receptor kinase inhibitor K252a (0.1 μM, Sigma, USA) for 24 hours.[26]

Mimics control (NC mimics): 5′ UUG UAC UAC ACAAAA GUACUG 3′), miR-10a-5p mimic: 5′ UAC CCU GUAAU CCG AAU UUG UG 3′. BDNF for pcDNA3.0 (pc)-BDNF and pcDNA vector. MiR-10a-5p mimic, NC mimics, and pc-BDNF were obtained from GenePharma (Shanghai, China). Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Inhibitor control (NC inhibitor): 5′ CAG UAC UUU UGU AGU ACA A 3′, miR-10a-5p inhibitor: 5′ CAC AAA UUC GGA UCU ACA GGG UA 3′. MiR-10a-5p inhibitor and NC inhibitor were obtained from GenePharma. Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

### Dual Luciferase Reporter Assay

A fragment (5′UUGUAUAUAUACAUAACAGGGUA3′) of the BDNF 3′UTR containing the predicted binding site was synthesized, and inserted into the plasmid downstream of the luciferase reporter gene, and named as wild-type (WT)-BDNF. A BDNF 3′UTR fragment (5′UUGUAUAUAUACAUAUCUGCGAA3′) with mutant (Mut) sequence was synthesized, also inserted into the plasmid luciferase reporter vector and referred to as Mut-BDNF. Hep-2 and AMC-HN-8 cells were transfected with miR-10a-5p mimic

and then co-transfected with BDNF-wild type (wt) or BDNF-mutant (mut) using Lipofectamine 2000. The luciferase assay was analyzed by using a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol following 48 hours of transfected.

## Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated by using a TRIzol reagent kit (Invitrogen, Beijing, China) according to manufacturers' protocol. Reverse transcription was performed with PrimeScript RT reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol. The quantitative real time-polymerase chain reactions (qRT-PCR) were assembled using the 2 SYBR Premix Ex Taq™ II (Takara) and subjected to the following protocol in Bio-Rad CFX-96 (Bio-Rad, CA, USA): 30 seconds at 95°C, 40 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. The melting curve was performed from 65°C to 95°C in 1°C/10 s increments. GAPDH and U6 were used for normalizing, respectively. The qRT-PCR data were analyzed using $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of mRNA. miR-10a-5p-F:5′CGCTACCCTGTAGATCCGAA3′miR-10a-5p-R: 3′GTGCAGGGTCCGAGGT 5′TrkB-F:5′GAAAAGCAAAAACCCTGTCTAGA3′, TrkB-R:3′TTAGCACACTCCTGCCATT5′.

## Western Blot Assay

Proteins were extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Protein concentrations were detected using the BCA Protein Assay Kit. The quantity of protein was separated by 10% dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then subsequently were transferred into polyvinylidene difluoride (PVDF) membranes (Merck Millipore) by electro-blotting. The membranes were blocked with 5% skim milk in TBST for 1 hour, and then incubated with primary antibody anti-SOX2 (1:1000, #14,962, Cell Signaling), anti-OCT4 (1:1000, #2750, Cell Signaling), anti-BDNF (1:1000, #47,808, Cell Signaling), and anti-TrkB (1:1000, #4603, Cell Signaling) overnight at 4°C before subsequent incubation with a second antibody (Cell Signaling Technology) for 1 hour at 37°C. The band densities were determined and analyzed with an automatic digital gel image analysis system Bio-Rad CFX-96 (Bio-Rad, CA, USA).

## Colony Formation Assay

Hep-2 and AMC-HN-8 cells of the control group, shRNA-NC group, TrkB-shRNA1 group, K252a group, mir-10a-5p group inhibitor, and TrkB-shRNA1 + inhibitor were pretreated with PBS. After that, the cell was cultured in a 6-well plate with 500 cells per well for 14 days. After that, the medium was discarded, and the cell was fixed with ethanol for 30 minutes and stained with 0.5% crystal violet. Finally, the cell was rinsed with deionized water to dry, and the photo was taken for observation.

## Flow Cytometry Analysis

Flow cytometry was performed to detect cell apoptosis. As previously described, Hep-2 and AMC-HN-8 cells were gently homogenized and harvested. Cells were re-suspended in EDTA-FEM at a density of $1\times10^5$, stained with Annexin V-FITC and PI, respectively. Finally, The sample was analyzed using a FACS Aria cell sorter (BD Biosciences). The data analysis was performed using Flow Jo (Tree Star, Ashland, OR).

## Immunohistochemistry

The tumor was fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin, and sectioned. The paraffin-embedded tumor were separated in xylene and rehydrated in gradient ethanol. After the antigen was extracted in 10 mM citric acid buffer, the tissue sections were incubated in 3% $H_2O_2$ for 10 minutes and sealed at room temperature for 1 hour. The tissue sections were then incubated overnight with rabbit anti-TrkB (1:2560, #4607, Cell Signaling) and anti-SOX2 (1:300, #14,962, Cell Signaling Technology). The corresponding second antibody (Cell Signaling Technology) was incubated at room temperature for 1 hour. The images were observed under an Olympus DX51 fluorescence microscope (Olympus, Tokyo, Japan). The data were analyzed by image 6.0.

## The Xenograft Tumor Model Assay

All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Affiliated Hospital of North Sichuan Medical College in 2019. The animal experiment was also approved by the institutional review board and the ethics committee of the Affiliated Hospital of Southwest Medical University (K2019182). The

orthotopic xenograft mouse models were established by using BALB/c athymic nude mice (female, 6-week-old). All animals were housed in the SPF environment and got unlimited water and food. $5.0\times10^6$ Hep-2 cells were injected into the lift flank leg of each nude mouse. They were randomly divided into two groups with treatment of 1) shRNA-NC, and 2) TrkB-shRNA1. Eight mice were included in each group for this experiment.

## TUNEL Staining

The heart tissues embedded in paraffin in different groups were sliced into sections (with a thickness of 3 μm) via the microtome. After routine deparaffinization, 50 μL 3% hydrogen peroxide solution was added and incubated at 20°C for 10 minutes, followed by rinsing with phosphate-buffered saline (PBS) 3-times. Then the sections were added into 50 μL reaction mixture of TUNEL and incubated at 37°C for 60 minutes. Next, 50 μL converter-peroxidase (POD) was added and incubated at 37°C for 30 minutes, followed by rinsing with PBS 3-times. Reagents A, B, and C in the Dolichosbiflorus agglutinin (DBA) kit were added into the sections for color development for 10 minutes. After that, the sections were washed with PBS 3-times, then counterstained with hematoxy[illegible] for 10 seconds and mounted in neutral balsam. The apoptotic cells stained yellowish brown were obs[illegible]d and counted under the microscope.[28] The ima[illegible]s were cap-tured by a special OLYMPUS DX51 fluo[illegible]scence [illegible] scope (Tokyo, Japan).

## Statistical Analysis

All results were presented [illegible]s mean±SD. T[illegible]-group comparisons were perform[illegible] with [illegible]udent's *t*-test. Multiple group comparisons [illegible]re analyzed with one-way ANOVA. Statisti[illegible]analys[illegible] was [illegible]rformed with SPSS software (ve[illegible]on 19[illegible] Values [illegible]<0.05 were considered statistically [illegible]gnific[illegible]

# Results

## Analysis of the Relationship Between TrkB and Clinicopathological Parameters in Patients with Laryngeal Cancer

In order to clarify the clinical significance of TrkB in laryngeal cancer, this study analyzed the relationship between TrkB and gender, age, smoking history, clinical stage, lymph node metastasis, and tumor site in patients with laryngeal cancer (Table 1). The result displayed that

**Table 1** Analysis of the Relationship Between TrkB and the Clinicopathological Parameters of the Patients with Laryngeal Cancer

| Group | Number (n) | TrkB | | *P*-value |
|---|---|---|---|---|
| | | positive (n) | Positive rate (%) | |
| Age (years) | | | | |
| <60 | 48 | 35 | 72.9 | 0.788 |
| ≥60 | 21 | 14 | 66.7 | |
| Gender | | | | |
| Male | 65 | 47 | [illegible] | 0.66 |
| Female | 4 | 2 | 50 | |
| Smoking history | | | | |
| Yes | 39 | [illegible] | [illegible]7 | 0.022 |
| No | 30 | 11 | 36.[illegible] | |
| Clinical stages | | | | |
| I–II | 3[illegible] | [illegible] | [illegible].4 | 0.015 |
| III–IV | [illegible] | 31 | 84.0 | |
| Lymphatic metastasi[illegible] | | | | |
| Tr[illegible]fer | 41 | 27 | 65.9 | 0.028 |
| [illegible]t transfer | [illegible] | 10 | 35.7 | |
| Tum[illegible]site | | | | |
| Supra[illegible]ic | [illegible]5 | 7 | 46.7 | 0.057 |
| Glottic | 24 | 13 | 54.2 | |
| [illegible] | 30 | 17 | 56.7 | |

rkB had nothing to do with the gender, age, or tumor location of patients with laryngeal cancer ($P>0.05$), but was related to smoking history, clinical stage, and lymph node metastasis ($P<0.05$).

## Relationship Between the Relapse-Free Survival Time, Total Survival Time, and Clinical Case Parameters in Patients with Laryngeal Cancer

In this study, a multivariate Cox proportional hazard model was used to analyze the relationship between recurrence-free survival time, total survival time, and clinical case parameters in patients with laryngeal cancer (Table 2). The result showed that recurrence-free survival time was not related to gender and T stage, related to age, N stage, and M stage. Overall survival time was not related to gender, age, T stage, and N stage, and is related to M stage and TrkB expression. TrkB-positive patients and TrkB-negative patients had significant differences in relapse-free survival and overall survival.

**Table 2** Analysis of Multivariable Cox Proportional Risk Model

| Parameter | RFS | | OS | |
|---|---|---|---|---|
| | HR (95% CI) | P[a] | HR (95% CI) | P[a] |
| Sex: Men vs women | 330,329.858 (0.000, -) | 0.987 | 1.615 (0.376–6.927) | 0.519 |
| Age: ≥60 vs <60 years | 0.321 (0.119–0.863) | 0.024 | 0.876 (0.468–1.637) | 0.678 |
| Tumor classification: T1, T2, T3, T4 | 1.158 (0.651–2.061) | 0.618 | 1.122 (0.707–1.780) | 0.625 |
| Lymph node status: N0, N1, N2, N3 | 5.840 (1.708–19.972) | 0.005 | 1.889 (0.771–4.631) | 0.164 |
| Metastasis status: 1 vs 0 | 40.805 2.715–613.175) | 0.007 | 11.314 (1.340–95.499) | 0.026 |
| Markers: (+) vs (-) | 50.130 (1.744–1440.574) | 0.022 | 3.685 (1.068–12.717) | 0.039 |

**Note:** [a]P<0.05.

## Effects of Down-Regulation of TrkB on Hep-2 Cell Proliferation and Apoptosis

As a result, Figure 1A and B denoted the mRNA expressive levels of TrkB were up-regulated in tumor tissues compared with the normal group, at the same time, TrkB levels were up-regulated in cancer cell lines compared with NP69 cells, especially Hep-2 and AMC-HN-8 cells. Three kinds of shRNAs were designed to interfere with expression levels of TrkB, and then were transfected with laryngeal cancer cell line Hep-2 and AMC-HN-8 cells. The relative mRNA levels of TrkB were reduced in transfection with shRNAs group compared with control group, especially TrkB-shRNA1 (Figure 1C). In the meantime, the relative mRNA levels of TrkB were also reduced in transfection with inhibitor K252a group compared with control group (Figure 1D). Colony formation assay showed that the down-regulation of TrkB inhibited colonies number of Hep-2 and AMC-HN-8 cells compared with control group (Figure 1E and F, $P<0.05$). Flow cytometry exhibited that the down-regulation of TrkB promoted Hep-2 and AMC-HN-8 cell apoptosis compared with the control group (Figure 1G and H, $P<0.05$). It is demonstrated BDNF/TrkB protected various tumor cells from apoptosis.[15] These findings indicated that down-regulation of TrkB could inhibit the cell proliferation and promote cell apoptosis in vitro.

## Effects of Down-Regulation of TrkB on Hep-2 Cancer Stem Cell-Like Property

To further confirm whether down-regulated expression of TrkB inhibited cancer stem cell-like property in Hep-2 and AMC-HN-8 cells, as shown in Figure 2A–D, compared to the control group, the diameter of spheres and the number of spheres rates were decreased in down-regulation of TrkB ($P<0.05$). CD44 expression was inhibited in down-regulation of TrkB (Figure 2E and F, $P<0.05$). The protein expressive levels of cancer stem cells marker SOX2 and OCT4 were restrained in down-regulation of TrkB (Figure 2G and H, $P<0.05$). These results confirmed the down-regulation of TrkB could inhibit cancer stem cell-like property in vitro.

## MiR-10a-5p Regulated TrkB Expression by Interacting with 3′-UTR of BDNF

As a result, Figure 3A and B denoted the mRNA expressive levels of miR-10a-5p were down-regulated in tumor tissues compared with the normal group; at the same time, miR-10a-5p was down-regulated in cancer cell lines compared with NP69 cells, especially Hep-2 and AMC-HN-8 cells. The miR-NC and miR-10a-5p mimic transfections were performed to Hep-2 and AMC-HN-8 cells, respectively. The relative expression levels of miR-10a-5p were markedly increased compared with the control group (Figure 3C, $P<0.05$). The dual-luciferase reporter assay was carried out on the Hep-2 and AMC-HN-8 cells, and luciferase activity was inhibited significantly in co-transfected with the wild-type BDNF 3′-UTR and miR-10a-5p (Figure 3D and E, $P<0.05$). The resulting BDNF as a potential target of miR-10a-5p has been demonstrated.[16,17] The inhibitor-NC and miR-10a-5p inhibitor transfection were performed to Hep-2 and AMC-HN-8 cells, respectively. The relative expression levels of miR-10a-5p were markedly decreased in the inhibitor group compared with the control group (Figure 3F, $P<0.05$). As shown in Figure 3G, the protein expressive levels of BDNF and TrkB were significantly down-regulated in the TrkB-shRNA1 group compared with the control group ($P<0.05$), while the levels of BDNF and TrkB were up-regulated in the miR-10a-5p inhibitor group ($P<0.05$). However, the levels of BDNF and TrkB were notably down-regulated in the co-transfection TrkB-shRNA1 + miR-10a-5p inhibitor group contrasted with the miR-10a-5p inhibitor group ($P<0.05$). Colony formation

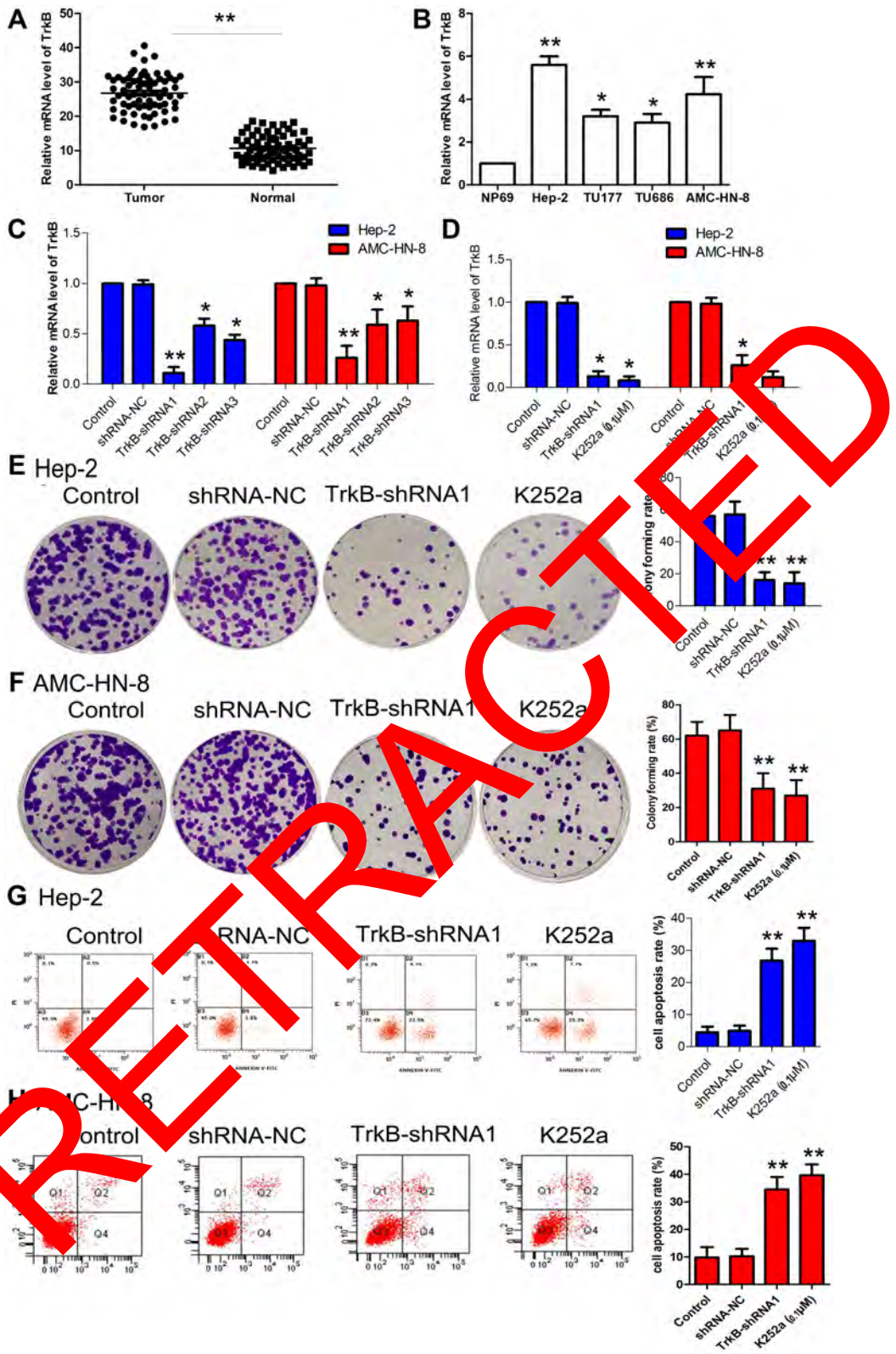




**Figure 1** Effects of down-regulation of TrkB on Hep-2 cell proliferation and apoptosis. (**A**) The mRNA levels of TrkB were detected by qRT-PCR in laryngeal cancer patient. (**B**) The mRNA levels of TrkB were detected by qRT-PCR in laryngeal cancer cell lines Hep-2, TU177, TU686, and AMC-HN-8. (**C**) Relative expression levels of TrkB in Hep-2 and AMC-HN-8 cells were transfected with shRNAs, namely shRNA1, shRNA2, and shRNA3. TrkB expression was examined by qRT-PCR. (**D**) Relative expression levels of TrkB in Hep-2 and AMC-HN-8 cells were transfected with shRNA-NC, TrkB-shRNA1, and K252a. (**E**) Hep-2 cells proliferation was performed by colony formation assay. (**F**) AMC-HN-8 cells proliferation was performed by colony formation assay. (**G**) Hep-2 cells apoptosis was performed by flow cytometry. (**H**) AMC-HN-8 cells apoptosis was performed by flow cytometry. The results were presented as mean±SD and represent three individual experiments. (*$P$<0.05, **$P$<0.01, compared with the control group).

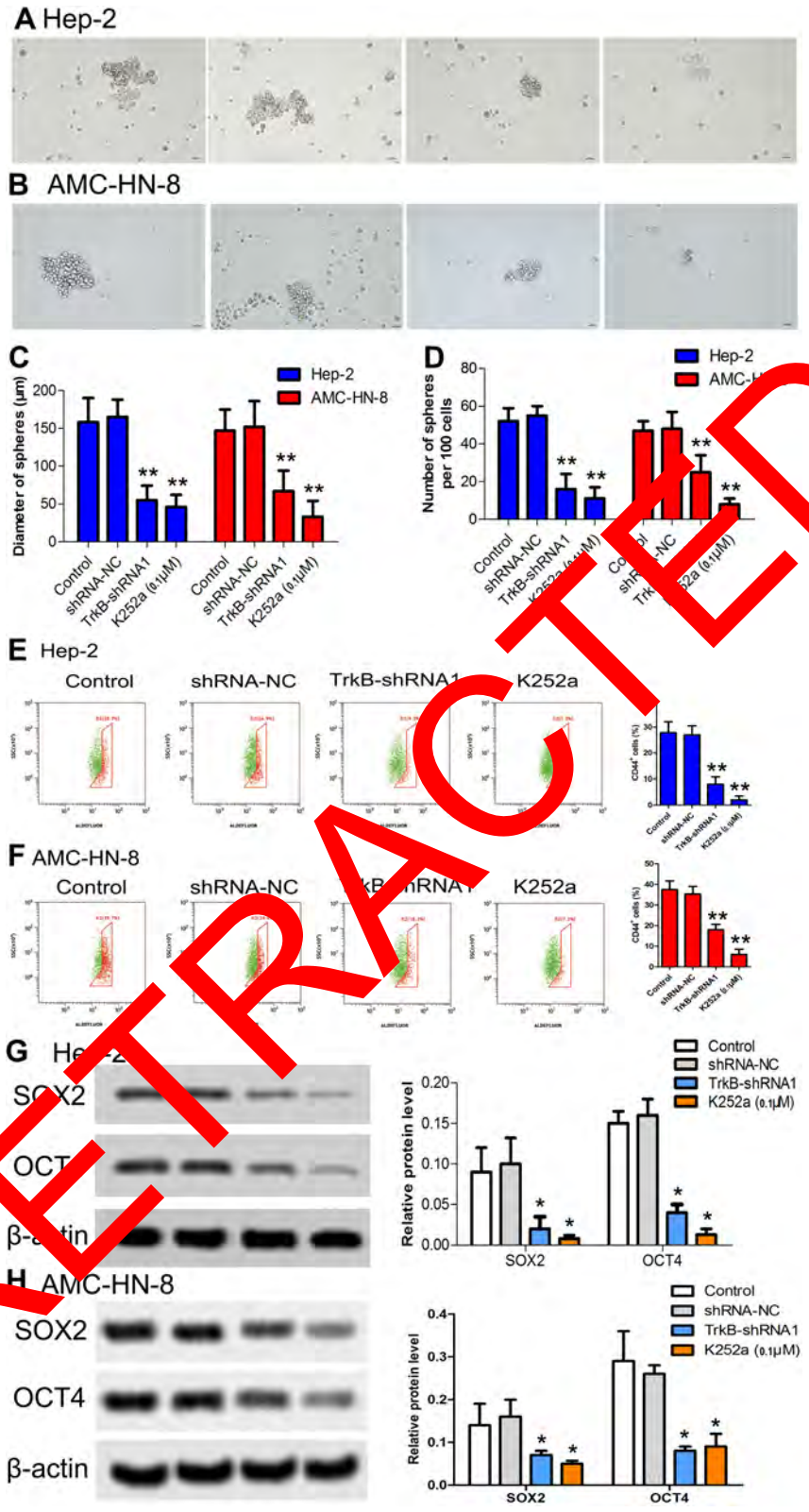




**Figure 2** Effects of down-regulation of TrkB on Hep-2 cancer stem cell-like property. (**A**) Hep-2 cells were cultured in a 37°C incubator containing 5% $CO_2$. (**B**) AMC-HN-8 cells were cultured in a 37°C incubator containing 5% $CO_2$. (**C**) Diameter of spheres in both Hep-2 and AMC-HN-8 cells. (**D**) Stem cell number of nodes was measured by electron microscope in both Hep-2 and AMC-HN-8 cells. (**E**) CD44 expression was performed by flow cytometry in Hep-2 cells. (**F**) CD44 expression was performed by flow cytometry in AMC-HN-8 cells. (**G**–**H**) The protein expressive levels of cancer stem cells marker SOX2 and OCT4 were detected by Western blot in both Hep-2 and AMC-HN-8 cells. Semi-quantitative analysis of the relative protein levels in each group. The results were presented as mean±SD and represent three individual experiments. (*$P$<0.05, **$P$<0.01, compared with the control group).

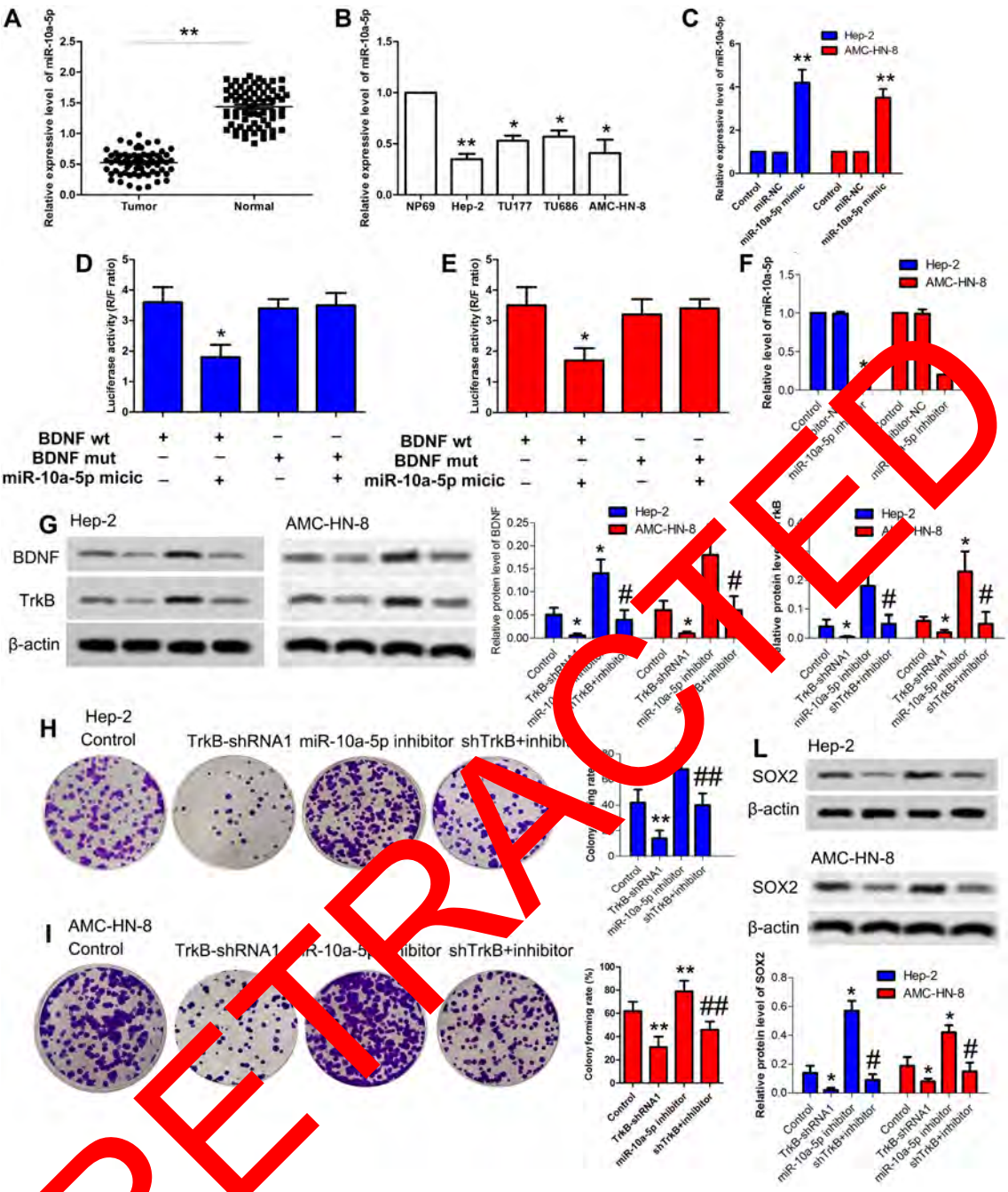




**Figure 3** MiR-10a regulated TrkB expression by interacting with 3′-UTR of BDNF. (**A**) The mRNA levels of miR-10a-5p were detected by qRT-PCR in laryngeal cancer patients. (**B**) The mRNA levels of miR-10a-5p were detected by qRT-PCR in laryngeal cancer cell lines Hep-2, TU177, TU686, and AMC-HN-8. (**C**) Relative expression level of miR-10a-5p in both Hep-2 and AMC-HN-8 cells were examined by qRT-PCR, including control, miR-NC, and miR-10a-5p mimic. (**D**) Luciferase activity was presented relative to that of control and mutant or wild-type BDNF3′-UTR in Hep-2 cells. Quantitation of signal intensities was performed by Image J software. (**E**) Luciferase activity was presented relative to that of control and mutant or wild-type BDNF3′-UTR in AMC-HN-8 cells. Quantitation of signal intensities was performed by Image J software. (**F**) Relative expression level of miR-10a-5p in both Hep-2 and AMC-HN-8 cells were examined by qRT-PCR, including control, inhibitor-NC, and miR-10a-5p inhibitor. (**G**) The protein expressive levels of BDNF and TrkB were detected by Western blot in both Hep-2 and AMC-HN-8 cells. Semi-quantitative analysis of the relative protein levels in control group, TrkB-shRNA1 group, miR-10a-5p inhibitor group, and TrkB-shRNA1 + inhibitor group. (**H**) Hep-2 cells proliferation was performed by colony formation assay. (**I**) AMC-HN-8 cells proliferation was performed by colony formation assay. (**L**) The protein expressive levels of SOX2 were detected by Western blot in both Hep-2 and AMC-HN-8 cells. Semi-quantitative analysis of the relative protein levels in each group. The results were presented as mean±SD and represent three individual experiments. (**P*<0.05, ***P*<0.01, compared with the control group, #*P*<0.05, ##*P*<0.01, compared with the miR-10a-5p inhibitor group).

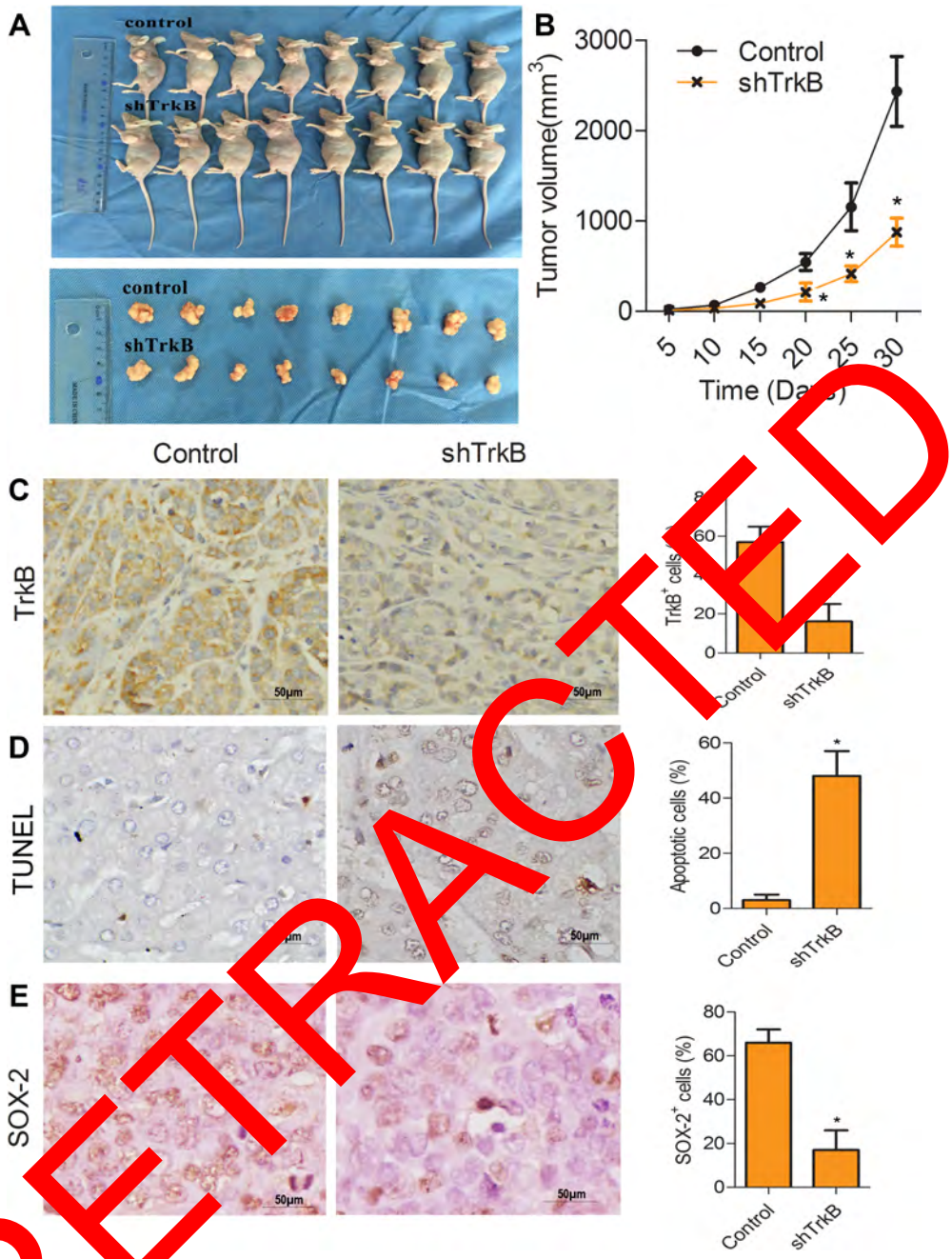




**Figure 4** TrkB-shRNA1 inhibited the tumor growth in vivo. (**A**) The tumor weight was determined when the mice were killed at the 30th day. (**B**) The broken line graph showed tumor weight (g). (**C**) Immunohistochemistry analysis of expression of TrkB and SOX2. Magnification, ×400. (**D**) Apoptosis was determined by TUNEL. Magnification, ×400. The results were presented as mean±SD and represent three individual experiments. (*$P$<0.05, compared with control group).

assay showed the colony formation rate was reduced in the TrkB-shRNA1 group compared with the control group ($P$<0.05) and elevated in the miR-10a-5p inhibitor group ($P$<0.05). Interestingly, the colony formation rate was reduced contrasted with the miR-10a-5p inhibitor group when TrkB-shRNA1 and miR-10a-5p inhibitors were co-transfected with Hep-2 and AMC-HN-8 cells (Figure 3H and I, $P$<0.05). The protein expression levels of SOX2 were decreased in the TrkB-shRNA1 group with increases in the miR-10a-5p inhibitor group (P<0.05). Nevertheless, when the TrkB-shRNA1 and miR-10a-5p inhibitor were co-transfected with Hep-2 and AMC-HN-8 cells, the levels of SOX2 were lower than the inhibitor group (Figure 3L, $P$<0.05). These results indicated that miR-10a-5p could mediate expression of TrkB via regulating BDNF.

## TrkB-shRNA1 Inhibited the Tumor Growth in vivo

To confirm the effect of TrkB-shRNA1 for tumor growth, as shown in Figure 4A and B, xenograft tumor volume was declined notably in vivo compared with the non-transfection TrkB-shRNA1 group ($P<0.05$). The protein expressive levels of TrkB and SOX2 were down-regulated markedly in vivo compared with the non-transfection TrkB-shRNA1 group ($P<0.05$) from immunohistochemical (Figure 4C and E). TUNEL assay displayed that cell apoptosis was enhanced in vivo compared with the non-transfection TrkB-shRNA1 group (Figure 4D, $P<0.05$). These results confirmed that TrkB-shRNA1 could inhibit the tumor growth in vivo.

# Discussion

Laryngeal cancer is one of the most common malignant tumors of the head and neck, accounting for about 1–5% of the systemic tumors. The pathological type is squamous cell carcinoma, which accounts for 93–99% of all laryngeal cancers. In northern China, the incidence of laryngeal cancer is higher, with the age of onset of 50–60 years. Importantly, the incidence of men is higher than that of women.[2] Trks are novel oncogenes involved in the formation of blood vessels, tumor progression, and lymph node metastasis in oral squamous cell carcinoma.[18] The existence of overexpressed BDNF and TrkB in human transitional cell carcinoma has been demonstrated.[19] However, the differences between tumors with high and low TrkB-expressing have not been fully described. High levels of TrkB-T1 mRNA are a unique squamous cell carcinoma subtype, enriched Nfe2l2 response, retinol metabolism, and hedgehog signaling pathways relevant to tumor progression.[20] This study has displayed that TrkB is related to smoking history, clinical stage, and lymph node metastasis, but has nothing to do with the gender, age, or tumor location of patients with laryngeal cancer.

The levels of TrkB are overexpressed in laryngeal cancer. TrkB signaling is involved in tumorigenicity of laryngeal cancer.[14] The up-regulated expression of BDNF and TrkB promote bladder cancer cell viability, proliferation, migration, and inhibit apoptosis.[12] Importantly, studies with head and neck squamous cell carcinoma cell lines have revealed that overexpression of BDNF promotes the cancer cell migration and invasion, but down-regulation of TrkB reverses this phenomenon.[21] In the research, down-regulation of TrkB inhibited the colonies number of Hep-2 and AMC-HN-8 cells, and promoted Hep-2 and AMC-HN-8 cells apoptosis. These findings have indicated that down-regulation of TrkB inhibit the cell proliferation and promote cell apoptosis in vitro.

Cancer stem cells (CSCs) are believed to be a small subpopulation of tumor cells that have properties of tumorigenesis, multilineage differentiation potential, self-renewal, slow cycling capacity, and tumorigenicity.[22] Some experimental results have indicated that CD133 is one of the markers for laryngeal carcinoma stem cells.[23] CD133 and CD44 expression, and aldehyde dehydrogenase (ALDH) activity are the CSCs markers that have been identified in laryngeal squamous cell carcinoma.[24] The CD44 expression was inhibited in down-regulation of TrkB. Moreover, the protein expressive levels of cancer stem cells marker SOX2 and OCT4 were restrained in Hep-2 and AMC-HN-8 cells. These results have confirmed the down-regulation of TrkB inhibit cancer stem cell-like property in vitro.

Although radical surgery and radiotherapy have been extensively used in the treatment of laryngeal cancer, local recurrence and tumor metastasis are also considered to be major challenges.[25] Previous research has demonstrated miR-10a-5p inhibits protein translation of BDNF, through binding to the BDNF.[16] MiR-10a-5p suppresses cancer proliferation and division in human cervical cancer by targeting BDNF.[17] In this research, the colony formation rate was reduced and contrasted with the miR-10a-5p inhibitor group when TrkB-shRNA1 and miR-10a-5p inhibitor were co-transfected with Hep-2 cells. When TrkB-shRNA1 and miR-10a-5p inhibitor were co-transfected with Hep-2 and AMC-HN-8 cells, the levels of SOX2 were lower than the inhibitor group. These results have indicated that miR-10a-5p mediate expression of TrkB in Hep-2 cells via regulating BDNF.



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## Disclosure

The authors report no conflicts of interest for this work.

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