Article

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# CD271 Serves as a Marker for Tumor-Initiating Cells in Laryngocarcinoma

Yupeng Shen[1,*], Chenzhi Xu[2], Xin Liu[1], Jihua Zhang[1], Fang Geng[1], Ruifeng Zhang[1], Dan Li[1], Bohao Zhang[1], Xiaoming Li[3]

[1]Department of Otorhinolaryngology Head and Neck Surgery, The First Hospital of Hebei Medical University, 050082 Shijiazhuang, Hebei, China
[2]Department of Otolaryngology, EYE & ENT Hospital, Fudan University, 200031 Shanghai, China
[3]Department of Otorhinolaryngology Head and Neck Surgery, Bethune International Peace Hospital, 050081 Shijiazhuang, Hebei, China
*Correspondence: fmmusypsyp@163.com (Yupeng Shen)
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**Background: Tumor-initiating cells (TICs) play a pivotal role in the unfavorable outcomes of laryngeal tumor proliferation, recurrence, and resistance to chemoradiotherapy. This study aims to explore the expression of CD271 (p75 neurotrophin receptor ($p75^{NTR}$) in human laryngocarcinoma Hep2 cells and unravel its potential biological functions as a marker of laryngeal TICs.**
**Materials and Methods: Immunomagnetic cell sorting was utilized to separate subsets of Hep-2 cells based on high and low expression levels of CD271. Various aspects such as proliferation activity, colony formation ability, cell cycle distribution, and the expression of cancer-related proteins in each subpopulation were evaluated using immunofluorescence, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, soft agar gel assay, flow cytometry, and western blot assay. Furthermore, the tumor-forming potential of the subsets displaying high and low CD271 expression was examined through an *in vivo* experiment involving nude mice. The proteins associated with the phosphorylated signal transducer and activator of transcription 3 (p-STAT3)/Octamer-binding transcription factor 4 (OCT4) pathway were detected via western blot assay.**
**Results: The expression of CD133 was the highest in the CD271 high-expression group, and the expression of CD133 was the lowest in the CD271 low-expression group. Hep2 cells with high CD271 expression exhibited enhanced proliferation capacity, in contrast to those with low CD271 expression which showed reduced proliferation ($p < 0.05$). The CD271 high-expression group of Hep2 cells demonstrated superior clonogenic ability, a higher proportion in the S and G2/M phases of the cell cycle, and an increased sphere-forming capacity. Moreover, Hep2 cells with high CD271 expression displayed enhanced tumor formation capability in nude mice ($p < 0.001$). Western blot analysis indicated significantly elevated levels of specific proteins such as OCT4, Nanog Homeobox (NANOG) and p-STAT3/STAT3 in the CD271 high-expression group were significantly higher than those in the control group ($p < 0.01$), and the protein levels of low-expression group were significantly lower than those in the control group ($p < 0.01$).**
**Conclusions: CD271 serves as a marker for TICs in Hep-2 cells, presenting a novel target for further investigation.**

**Keywords:** CD271 ($p75^{NTR}$); laryngocarcinoma; Hep-2; CD133

## Introduction

Laryngocarcinoma, a prevalent malignant tumor, exhibits notable invasiveness, a high recurrence rate, and resistance to chemotherapy, presenting significant challenges in clinical treatment [1]. This is particularly evident in advanced and recurrent cases, where exclusive reliance on surgical intervention often falls short of achieving complete eradication, leaving patients with compromised postoperative quality of life [2]. Recent investigations propose a correlation between the high recurrence rates observed in head and neck squamous cell carcinoma and the resistance to radiotherapy and chemotherapy, highlighting the involvement of tumor-initiating cells (TICs) or stem cells [3]. These cells, resistant to complete removal through surgery or conventional treatments, serve as the underlying cause of recurrence and distant metastasis [4,5]. Exploring effective methods to identify and selectively target these TICs represents a novel and crucial avenue in advancing therapies for laryngocarcinoma [6].

CD271, also known as the nerve growth factor low-affinity receptor p75 (p75 neurotrophin receptor ($p75^{NTR}$)), is a member of the Tumor Necrosis Factor Receptor (TNFR) family, with widespread distribution in both the nervous system and non-neural tissues, including tumor cells [7]. In most cells, CD271 shows a relatively equal affinity for various neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3/4 [8]. Recent research has revealed the complex biological functions of CD271, involving roles in cellular survival, proliferation, differentiation, migration,

and apoptosis [9]. The role of CD271 in tumorigenesis and progression has garnered increasing attention from researchers. Its functions appear to be context-dependent, varying among different cell types, developmental stages, and local environments, sometimes even showing opposing effects [9].

Initial investigations have revealed that CD271 expression is confined to the basal cells of normal laryngeal epithelia, aligning with the staining patterns characteristic of epithelial stem cells [10]. In human laryngeal squamous cell carcinoma (LSCC), CD271 exhibits differential expression. In well-differentiated LSCC, positive CD271 staining is primarily observed in the basal region and infiltrative margins surrounding cancer nests, indicative of cells with heightened proliferation potential or self-renewal properties. Conversely, in moderately or poorly differentiated LSCC, CD271-positive cells are diffusely distributed across a broader range [10]. This aberrant expression and distribution of CD271 may signify malignant transformation. TICs in head and neck squamous cell carcinoma (HNSCC) are identified by the surface expression of CD44 or CD133. Due to the heterogeneity of cancer, one of the major clinical challenges in treatment development is the ability of cancer to develop resistance. It is hypothesized that cancer stem cells (CSCs) are responsible for this resistance and that targeting them will cause tumors to regress [3]. The use of the pentterpenoid transmembrane glycoprotein CD133 has been proposed to label CSCs of various tumor types and high expression of CD133 in tumors has been indicated as a prognostic marker of disease progression. Activation of CD271-mediated downstream signals requires the expression of specific environmental signals by TICs. These specific environmental signals link the development stage to the local environment in different cell types. Murillo-Sauca *et al*. [11] research suggests that CD271 more specifically identifies the TIC subpopulation within the $CD44^+$ compartment in HNSCC and functions as an active and targetable molecule. Currently, there is limited information available on the expression and role of CD271 in laryngeal carcinoma and its corresponding cell lines.

Signal transducer and activator of transcription 3 (STAT3) is an important transcription factor, which plays a key role in the regulation of cell growth, differentiation, survival, and metabolism [12]. STAT3 also plays an important role in promoting tumor stemness [13]. STAT3 promotes the expression of genes related to CSC properties, such as promoting self-renewal and multi-directional differentiation potential of tumor cells.

In this study, we aimed to investigate the expression of CD271 ($p75^{NTR}$) in human laryngocarcinoma Hep2 cells and explore its potential molecular mechanisms and biological functions as a marker of laryngeal TICs.

## Materials and Methods

### *Tissue Specimens*

Tissue specimens were obtained from 42 randomly selected patients with primary T1–T4 LSCC after informed consent. All of these tumors were surgically resected during the period from 2008 to 2009 and were classified according to the sixth edition of the pathologic tumor-node-metastasis classification of UICC (2002). The diagnosis of squamous cell carcinoma was confirmed by a pathologist and was graded into well, moderately, or poorly differentiated in a blind pattern according to the World Health Organization (WHO) (Head and Neck Tumor) classification (2005).

### *Immunohistochemical Staining*

For immunohistochemical (IHC) staining, paraffin sections were cut serially at 4 µm, dried for 50 min at 60 °C, and incubated overnight at 37 °C. Sections were dewaxed with xylene, rehydrated through a graded alcohol series, and then treated with 0.3% $H_2O_2$ in methanol to inactivate endogenous peroxidase. Epitope retrieval was induced by heating the sections in citrate buffer (pH 6.0) for 3 min in an autoclave at 96 °C. After cooling to room temperature, the sections were washed with phosphate-buffered saline (PBS). Normal goat serum was used to eliminate nonspecific coloration. For immunocytochemical staining, cells on coverslips were fixed, and the endogenous peroxidase activity was achieved with 0.3% $H_2O_2$ in methanol (Beijing Chemical Reagent Factory, Beijing, China). Thereafter, the immunostaining was done with SP-9002 (Beijing Zhongshan Jinqiao Biotechnology Company, Beijing, China) kits following the manufacturer's instructions. Primary antibodies were diluted with 0.5% bovine serum albumin in PBS. The sections were incubated overnight at 4 °C with diluted primary antibody: 1:50 for rabbit anti-human CD271 monoclonal antibody (ab52987; Abcam, Cambridge, UK). After washing, the sections were incubated for 30 min with the appropriate secondary biotinylated antibody preparation (goat anti-rabbit Immunoglobulin G (IgG), both diluted at 5 µg/mL, ab6721, Abcam). Following PBS washes, sections were incubated with the avidin-biotin complex conjugated to horseradish peroxidase (ABC-HRP) for 30 min, then washed, and finally stained. The IHC sections were further scanned with NanoZoomer S60 (Hamamatsu Photonics, Benzhou Island, Japan).

### *Cell Culture and Magnetic Bead Sorting*

The laryngocarcinoma Hep-2 Cells (CTCC-085CS) were obtained from the pathology laboratory of the American National Cancer Institute and received mycoplasma detection. Hep-2 cells were authenticated using standard short tandem repeat-based DNA profiling (**Supplementary File 1**). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) (Gibco, Thermo Fisher Scien-

tific, Waltham, MA, USA) culture medium (containing L-Glutamine and 5 ng/mL Nerve Growth Factor (NGF)) with 5% fetal bovine serum (FBS) (Procell, Wuhan, China) in an incubator (8000, Thermo Scientific, Logan, UT, USA) at 5% $CO_2$, saturated humidity, and 37 °C. CD271-positive and CD271-negative Hep2 cells were selected and collected using a magnetic bead sorting kit (130-042-102, Mini MACS, Miltenyi Biotec, Germany), and cultured overnight for antibody identification of immunofluorescence CD271 (ab52987, Abcam, Cambridge, UK) and CD133 (18470-1-AP, Proteintech, Wuhan, China). Normal Hep-2 cells were used as the control group.

### *CD271 Immunoreactive Staining*

CD271 high-/low-expression Hep-2 cells were inoculated on microscope slides and fixed with 4% paraformaldehyde to maintain the integrity of their morphology and structure. Samples were incubated with a blocking solution of 10% serum to reduce non-specific binding. Primary antibody incubation: A specific primary CD271 antibody (1:100; ab52987; Abcam, Cambridge, UK) was incubated with the sample to bind specifically to the target antigen. Unbound primary antibodies were washed off with PBS to minimize background noise. Samples were then incubated with fluorescently labeled secondary antibodies. The samples were washed again to remove unbound secondary antibodies (goat anti-rabbit Immunoglobulin G (IgG), both diluted at 5 µg/mL, ab6721, Abcam). The nuclei were stained with fluorescent dyes such as 4',6-Diamidino-2-phenylindole (DAPI) to facilitate the observation of cell morphology and distribution under a microscope. Anti-quench agents were used to seal the slides to protect the fluorescent markers from photobleaching and to prolong the storage time of the samples. The expression of CD271 was analyzed by observing and recording fluorescence signals using a fluorescence microscope (DM150, Leica, Wetzlar, Germany).

### *Immunofluorescence*

High- and low-expression CD271 cells were grown separately in 96-well plates. When the cell density reached 30%, they were fixed with 4% paraformaldehyde for 30 minutes, and then 5% FBS (SH30396.03, Hyclone, Thermo Scientific, Logan, UT, USA) was sealed at room temperature for 1 hour. The first antibody (CD271 1:400, ab8874, Abcam; CD133 1:100, 18470-1-AP, Proteintech) was used to incubate overnight at 4 °C, and then the second antibody Cy3–conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (1:200, SA00009-2, Proteintech, Wuhan, China) was added. Cells were incubated at 37 °C for 1 hour, and the Hoechst was incubated in the dark at room temperature for 15 minutes.

### *3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay*

The CD271 high-expression and low-expression cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells/mL with 150 µL per well. Each group consisted of 5 replicate wells and was cultured in a 5% $CO_2$ incubator at 37 °C. Following incubation for 1, 2, 3, 5, and 7 days, 15 µL of MTT (M2128, Sigma, St. Louis, MO, USA) was added and the plates were incubated at 37 °C in a 5% $CO_2$ incubator (Germany8000) in the dark for 4 h. Subsequently, 150 µL of Dimethyl sulfoxide (DMSO) (D12345, Invitrogen, San Jose, CA, USA) was added, and the plates were shaken at room temperature for 10 minutes. The optical density (OD) value was measured at a wavelength of 492 nm using an enzyme marker to determine cell proliferation.

### *Soft Agar Colony-Formation Assay*

100 mL of 1.2% and 0.7% Agarose (G-10, Biowest, Nuaillé, France) was prepared with self-made double distilled water and samples were maintained at 42 °C to prevent them from solidifying. 1.2% Agarose, 20% FBS, 2 × RPMI1640 medium, and 2 × Antibiotics were mixed in a 1:1 ratio. 1.5 mL of mixed solution was added to each well in the 6-well plate and allowed to stand at room temperature until the lower layer of adhesive solidified. 0.7% agarose, 2 × RPMI1640 medium, and 2 × antibiotics were mixed in a 1:1 ratio, the cell suspension was added, mixed thoroughly, and then added to the 6-well plate covered with a 1.2% agarose layer, gradually forming a double agar layer.

### *Flow Cytometry Assay*

CD271 high- and low-expression cell populations were screened, by adding pre-cooled 75% ethanol after cell precipitation, fixing overnight at 4 °C, and adding 500 µL PBS containing 100 µg/mL RNase A (Sigma, USA), and incubated at 37 °C for 30 minutes, followed by the addition of Propidium Iodide (PI) (C1052, Beyotime Biotechnology, Shanghai, China) to achieve a concentration of 50 µg/mL. 200 µL Single cell suspension was incubated in the dark at 37 °C and was detected by flow cytometry (C6, BD Accuri™, USA) and analyzed by Cell Quest Software (C6, BD Accuri™, Franklin Lakes, NJ, USA).

### *Cell Cycle Assay*

A cell cycle staining kit was used for the determination of the cell cycle according to the instructions. Hep-2 cells were seeded in 24-well plates for 48 h. Cells were harvested and washed with precooled PBS twice, and then stained with 1 mL DNA staining solution and 10 µL permeabilization solution at room temperature for 30 min in the dark.

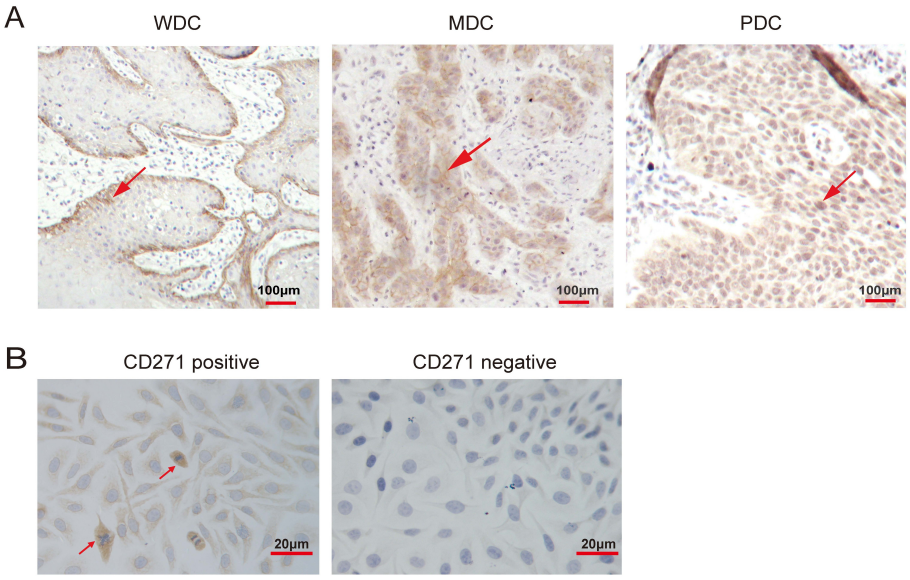


**Fig. 1. Immunohistochemical positive staining for CD271 in LSCC.** (A) Immunohistochemical (IHC) staining of CD271 in different differentiated laryngeal squamous cell carcinoma (LSCC) (3,3'-Diaminobenzidine (DAB)×200). WDC, well-differentiated LSCC; MDC, moderately differentiated LSCC; PDC, poorly differentiated LSCC. The red arrows indicate the positive cells. Scale bar: 100 μm. (B) IHC staining of CD271 in Hep2 cells (DAB×400); The red arrows indicate positive cells.

### *Serum-Free Screening of Stem Cell Spheres*

Low-adhesion culture plates were selected for cell inoculation. The screened CD271 high- and low-expressing cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, SH30023.01)/F12 medium containing 20 ng/mL Epidermal Growth Factor (EGF) (CYT-466, Prospec, East Brunswick, NJ, USA), 10 ng/mL Basic Fibroblast Growth Factor (BFGF) (Sigma, St. Louis, MO, USA), 2% B27 (Gibco), and 10 μg/mL Insulin (CYT-468, Prospec, USA), and induced into pellets; After pelletizing, the cells were distributed for passage and dry pelletizing was performed again. The spheroid formation of both cell types was observed microscopically by changing the solution every three days.

### *In vivo Tumor Model*

Female SPF nude mice (weight 20–25 g), 6–8 weeks old, were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China. All mice were provided with water and diet ad libitum and maintained in an animal house with constant temperature (23 $\pm$ 2 °C) and humidity. A total of 18 mice were randomly divided into 3 groups (6 per group). CD271 high-expression cells, CD271 low-expression cells, and normal Hep-2 cells were expanded and the cell concentration was adjusted to $5 \times 10^7$ cells/mL. Cells were collected and digested with trypsin. The mice were observed for 3 days without any abnormalities. After disinfecting the skin, the mice received a 5% subcutaneous injection into the forelimbs. The puncture point was about 1cm away from the injection point, forming a raised skin mound to prevent liquid leakage. Tumor formation was observed and the tumor size of each group of nude mice was measured and recorded twice a week. Cisplatin (Delivered Duty Paid (DDP, 2.5 mg/kg, HY-17394, MedChemExpress, Monmouth Junction, NJ, USA) and paclitaxel (TAX, 30 mg/kg, HY-B0015, MedChemExpress) were administered intraperitoneally when the tumor volume of the largest group was 200 $mm^3$, the tumors were removed and hematoxylin and eosin (H&E) staining was performed. Mice were euthanized by intramuscular injection of 100 mg/Kg sodium pentobarbital (3%, 2006006, New Asiatic Pharmaceutical, Shanghai, China).

### *Western Blot Analysis*

Cell protein was collected using Radio Immunoprecipitation Assay Lysis buffer (RIPA) lysate and the supernatant was taken for protein quantification using a Bicinchoninic Acid (BCA) protein quantification kit (BL521A, Beyotime Biotechnology). Samples (20 μg) were separated by Sodium Dodecyl Sulfate-polyacrylamide gel (SDS-PAGE), and then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (IPVH00010, Millipore, Burlington, MA, USA). The band position of the protein was marked and then the transfer film was fully sealed for 1 hour by applying Bovine Serum Albumin (BSA), and incubated overnight at 4 °C with the following primary antibodies: ACTIN (66009-1-Ig, Proteintech, used at 1:50,000), Nanog homeobox (NANOG) (ab80892, Abcam, used at 1:1000), STAT3 (ab68153, Abcam, used at 1:1000), p-STAT3 (ab76315, Abcam, used at 1:1000), and Octamer-binding tran-

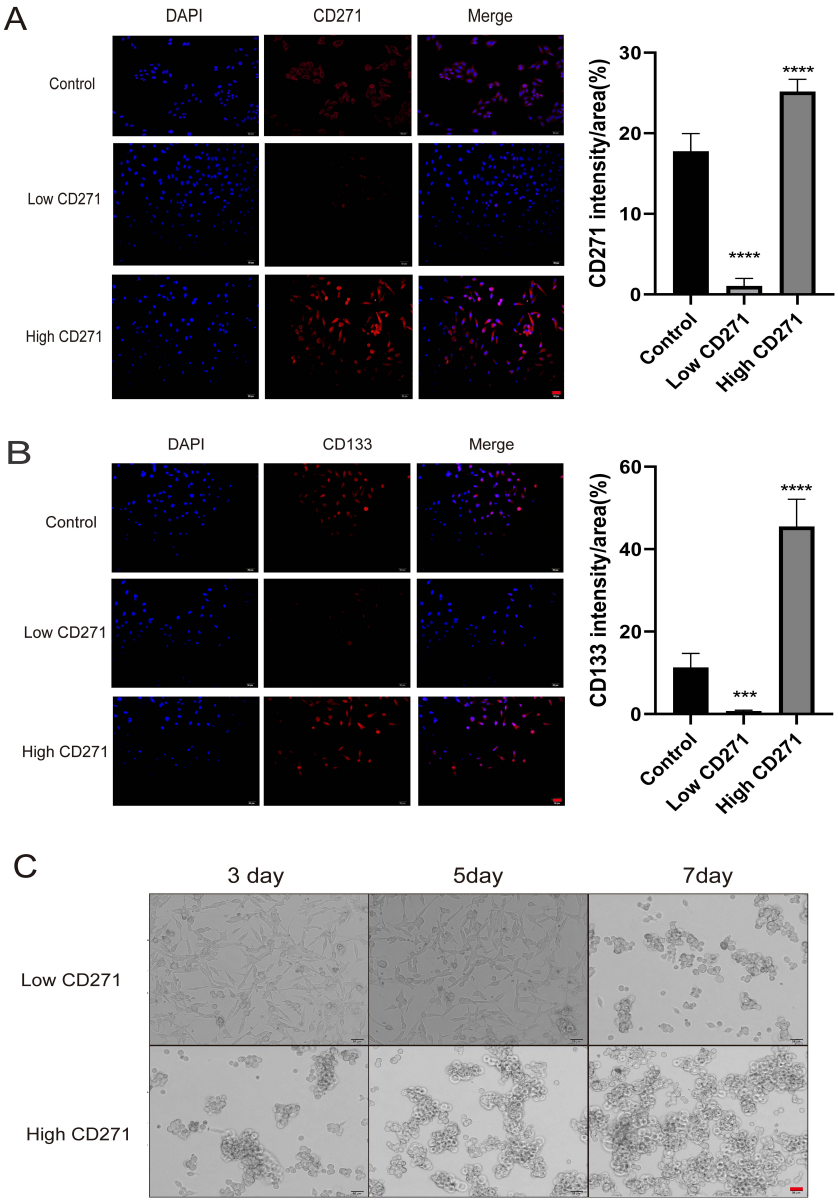


**Fig. 2. Immunofluorescence detection of sorted CD271 high- and low-expression cells and Serum-free screening for stem cell spheres.** (A) The expression of CD271 in the CD271 high- and low-expression groups was compared. (B) The expression of CD133 in the CD271 high- and low-expression groups was examined. (C) Serum-free screening for stem cell spheres in the CD271 high- and low-expression groups at 3 d, 5 d, and 7 d. ***$p < 0.001$, ****$p < 0.0001$. Scale bar: 20 µm. DAPI, 4',6-Diamidino-2-phenylindole.

scription factor 4 (OCT4) (ab18976, Abcam, used at 1:1000). Secondary antibody horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse polyclonal antibodies (HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L)(SA00001-1, Proteintech, used at 1:2000), HRP-conjugated Goat Anti-Rabbit IgG(H+L)(SA00001-2, Proteintech, used at 1:2000), incubated for 2 h at room temperature, were used for the detection of bound primary antibody, and bands were visualized by enhanced chemiluminescence. An Enzyme-Linked Chemiluminescence (eECL1) kit (E002-100, Qihai, Biotechnology, Shanghai, China) was used to conduct chemiluminescence detection. After developing and photographic fixing, photographs were taken and films were analyzed using the gel-imaging analytic system and the ImageJ 2.0 software (Rawak Software Inc., Stuttgart, Germany).

### *Statistical Analysis*

Statistical analysis was performed using SPSS 11.0 software (IBM-SPSS Statistics, Chicago, IL, USA). Continuous data are presented as the mean ± standard deviation (SD), and the differences among the experimental groups were analyzed using one-way Analysis of Variance (ANOVA) or Student's *t-test*. After ANOVA, multiple com-

parison tests were used to assess pairwise differences. We used the Bonferroni method for multiple comparisons correction. This test method has high sensitivity, as long as there is a small mean difference between groups. $p$ values $< 0.05$ were considered statistically significant.

## Results

### *Expression of CD271 in Different Differentiated Laryngeal Squamous Cell Carcinoma and Hep2 Cells*

We first examined the expression of CD271 in different differentiated LSCC by immunohistochemistry. We first examined the expression of CD271 in different LSCC (Laryngeal Squamous Cell Carcinoma) cell types by immunohistochemistry after they had undergone differentiation. As shown in Fig. 1A, CD271-positive staining was apparent in the basilar part and infiltrative margin of cancer nests where most of the cells were actively proliferating in well-differentiated LSCC (WDC); CD271 was expressed in a wider range from tumor cell clusters margins in moderately differentiated LSCC (MDC); The distribution of CD271 staining became more disseminated in poorly differentiated LSCC (PDC). Next, we conducted immunoreactive staining for laryngocarcinoma Hep2 cells by using the CD271 monoclonal antibody and found that some of the Hep2 cells (about 30–40%) displayed weak CD271-positive immunoreactivity, whereas a small fraction of Hep2 cells displayed strong CD271-positive immunoreactivity (about 4–6%), as shown in Fig. 1B. However, the biological significance and role of CD271-positive and CD271-negative Hep-2 cells remain unclear.

### *Hep-2 Cells with High Expression of CD271 had Significantly Increased Stemness*

We selected and collected CD271-positive (high) and CD271-negative (low) Hep2 cells using a magnetic bead sorting kit. Hep-2 cells were used as the control group. The immunofluorescence results showed that CD271 expression was significantly increased in the CD271 high/positive group compared with control, whereas CD271 expression was significantly decreased in the CD271 low/negative group compared with control group (Fig. 2A, $p < 0.0001$). In addition, we compared the CSC marker gene (CD133) expression between the CD271 high group and the CD271 low group. The expression of CD133 was highest in the CD271 high-expression group, whereas the expression of CD133 was lowest in the CD271 low-expression group (Fig. 2B, $p < 0.001$). Moreover, Serum-free screening of stem cell spheres showed that a small number of cells in the CD271 high-expression group had already formed spheres at 3 days. As time increased, the number and size of spheres also increased. The CD271 low-expression group still had not formed spheres at 3 and 5 days, and a small number of cells had begun to form spheres at 7 days. However, the number of spheres was small and the volume was small, so the sphere-forming rate of the CD271 high-expression group was higher than that of the CD271 low-expression group (Fig. 2C). Thus, CD271 serves as a marker for TICs in laryngocarcinoma.

### *Hep-2 Cells with High Expression of CD271 Significantly Increased Proliferation, and Decreased Cell Apoptosis*

The MTT results showed that the proliferation ability of CD271 high-expression cells was greater than that of CD271 low-expression cells (Fig. 3A, $p < 0.001$). In the control group, the detected OD value was intermediate, but by 7 days, the cells had entered the decay phase. The Soft Agar Clone experimental results showed that the CD271 low-expression group had almost no clone clusters at 3 days, the clone clusters were smaller at 5 days, and slightly larger at 7 days. The high-expression group of CD271 formed a small number of clone clusters at 3 days, and as time went on, the number of clone clusters increased (Fig. 3B). The Flow Cytometry experiment results showed that, within three groups, the CD271 high-expression group displayed a higher proportion of cells in S phase and G2/M phase, and a lower proportion in G0/G1 phase (Fig. 3C,D).

### *Hep-2 Cells with High Expression of CD271 Significantly Increased Tumorigenic Ability of Hep-2 Cells*

Next, we generated a subcutaneous xenograft tumor mouse model. Hep-2 cells of CD271 high expression, control, and CD271 low expression were subcutaneously injected into separate experimental groups of nude mice. The mice were administrated cisplatin (DDP, 2.5 mg/kg) and paclitaxel (TAX, 30 mg/kg). Results showed that the growth of CD271 high-expression Hep-2 cells induced xenografts that were than those of Hep-2 control cells, and the xenografts induced by CD271 low-expression Hep-2 cells were smaller than those of control Hep-2 cells under DDP and TAX treatments (Fig. 4A). Thus, CD271 high expression increased the tumor volume compared with the control groups (Fig. 4B,C, $p < 0.05$). There were significant differences in the growth of three groups induced xenografts. But after the addition of DDP and TAX chemotherapy drugs, the growth of tumors in all three groups was significantly inhibited. Finally, we evaluated the morphological alterations of tumors by H&E staining (Fig. 4D).

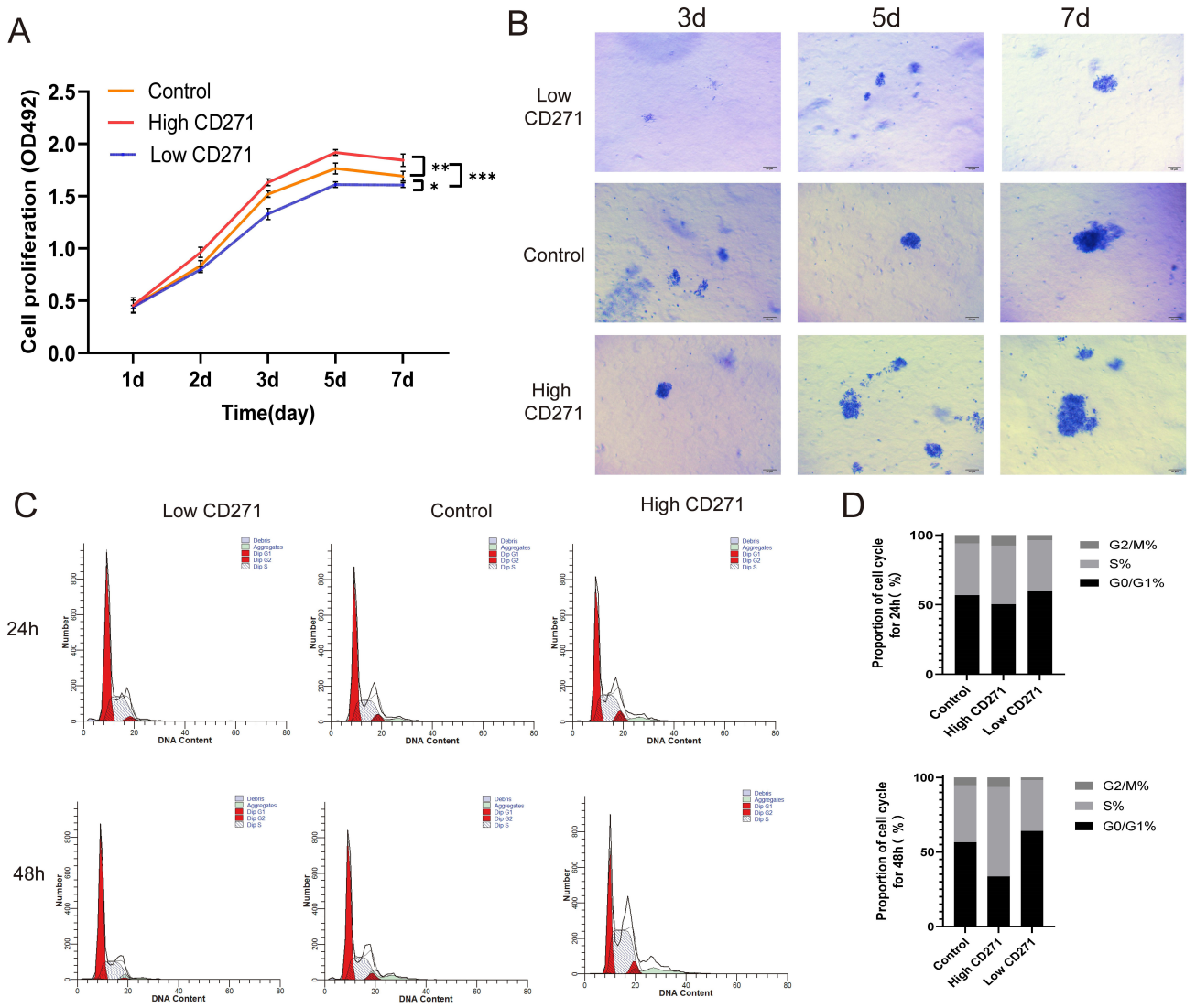


**Fig. 3. Effect of CD271 high and low expression on proliferation and apoptosis in Hep-2 cells.** (A) Cell proliferation in Hep2 cells with high and low CD271 expression was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n = 5. (B) Analysis of clonal formation of CD271 high- and low-expression cell populations was performed using soft agar clones. Scale bar: 50 µm. n = 3. (C) Flow cytometry was employed to compare the cell cycle distribution of cells in CD271 high- and low-expression groups. (D) The distribution of cells in the G0/G1, S, and G2/M phases was quantified for two time points.

### *The Protein Expression of STAT3, p-STAT3, OCT4, and NANOG was Upregulated in Hep-2 Cells with CD271 High Expression*

Cancer cells share some characteristics with embryonic stem cells, such as unlimited proliferation capacity, self-renewal, and expression of pluripotency genes (NANOG and OCT4). The protein expression levels of NANOG, OCT4, and p-STAT3/STAT3 were detected by western blotting assay in the Hep-2 cells with CD271 high expression, control, and low expression (Fig. 5A). Western blot experimental results showed that the protein levels of OCT4 (Fig. 5B), NANOG (Fig. 5C) and p-STAT3/STAT3 (Fig. 5D) in the CD271 high-expression group were significantly higher than those in the control group ($p < 0.01$), and the protein levels of low-expression group were significantly lower than those in the control group ($p < 0.01$).

## Discussion

Understanding the regulatory mechanisms influencing the onset, progression, and metastasis of laryngocarcinoma has emerged as a focal point in head and neck cancer research. Tumor initiation and growth within the human body environment necessitate regulatory support from numerous growth factors and their corresponding receptor signaling pathways. Neurotrophins possess two types of receptors: high-affinity binding tyrosine kinase recep-

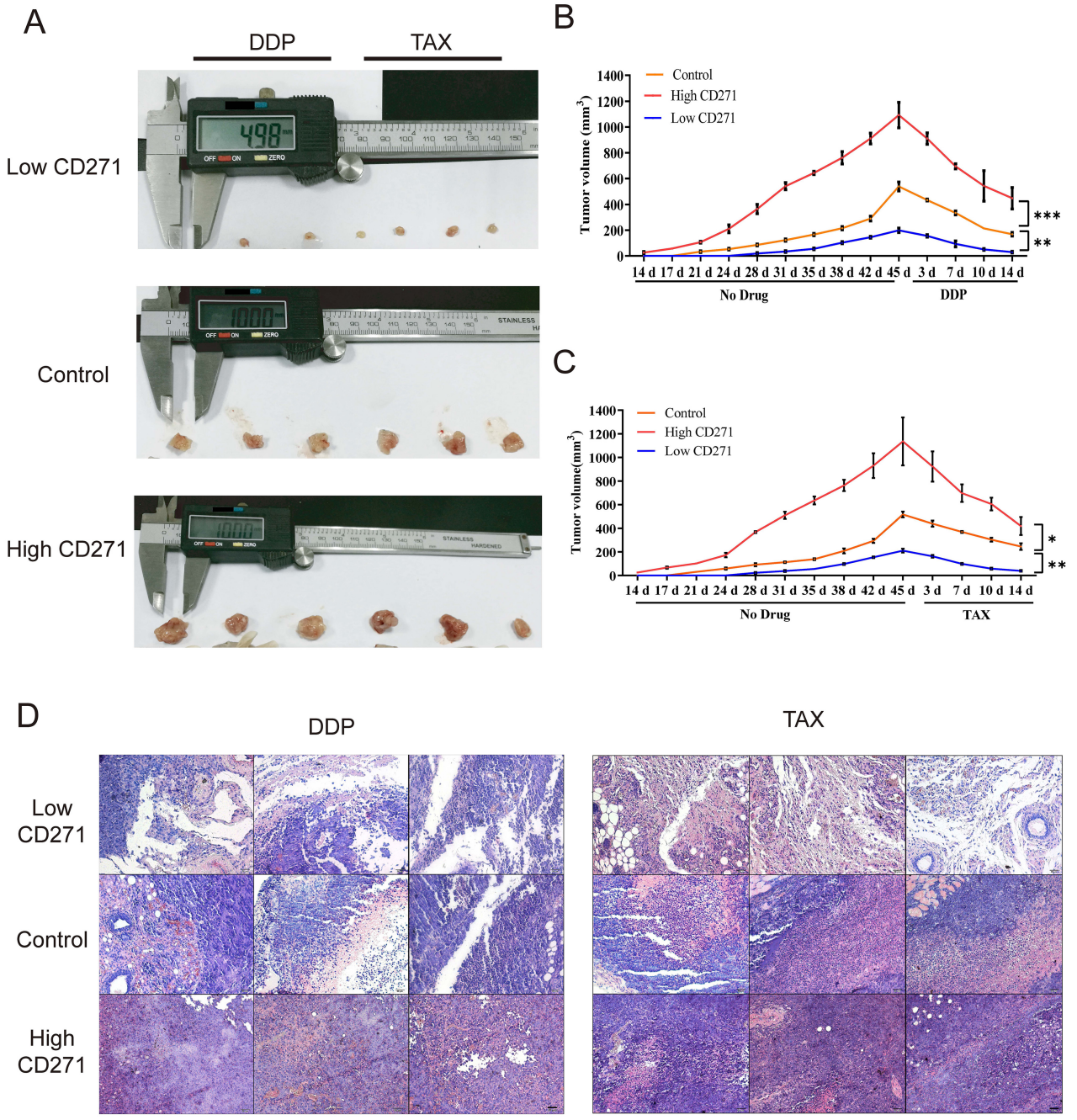


**Fig. 4. Tumor formation experiment in nude mice.** (A) The tumor size of CD271 high-expression, control, and CD271 low-expression groups under Delivered Duty Paid (DDP) and TAX treatment was compared. n = 3. (B) The tumor volume of control, CD271 high-expression, and CD271 low-expression groups under DDP treatment were compared. n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$. (C) The tumor volume of control, CD271 high-expression, and CD271 low-expression groups under TAX treatment were compared. n = 3. $^{*}p < 0.05$, $^{**}p < 0.01$. (D) Hematoxylin and eosin (H&E) staining of tumor tissues from the DDP and TAX treatment is shown (Scale bar: 20 µm). n = 3. The caliper shown in the image does not represent actual measurement data but is set to a fixed value to serve as a scale bar for reference to the tumor size.

tors (Receptor Tyrosine Kinases (RTK)/Tropomyosin receptor kinases (Trks)) and low-affinity binding p75 receptors (CD271). In recent years, the involvement of the CD271 receptor system in tumor onset and development has captured the attention of researchers. Extensive studies have been conducted on various tumors, including gastric cancer, bladder cancer, prostate cancer, breast cancer, and esophageal cancer, among others [14,15]. A growing body of evidence indicates that CD271 plays a pivotal role in tumor onset and progression [16]. The func-

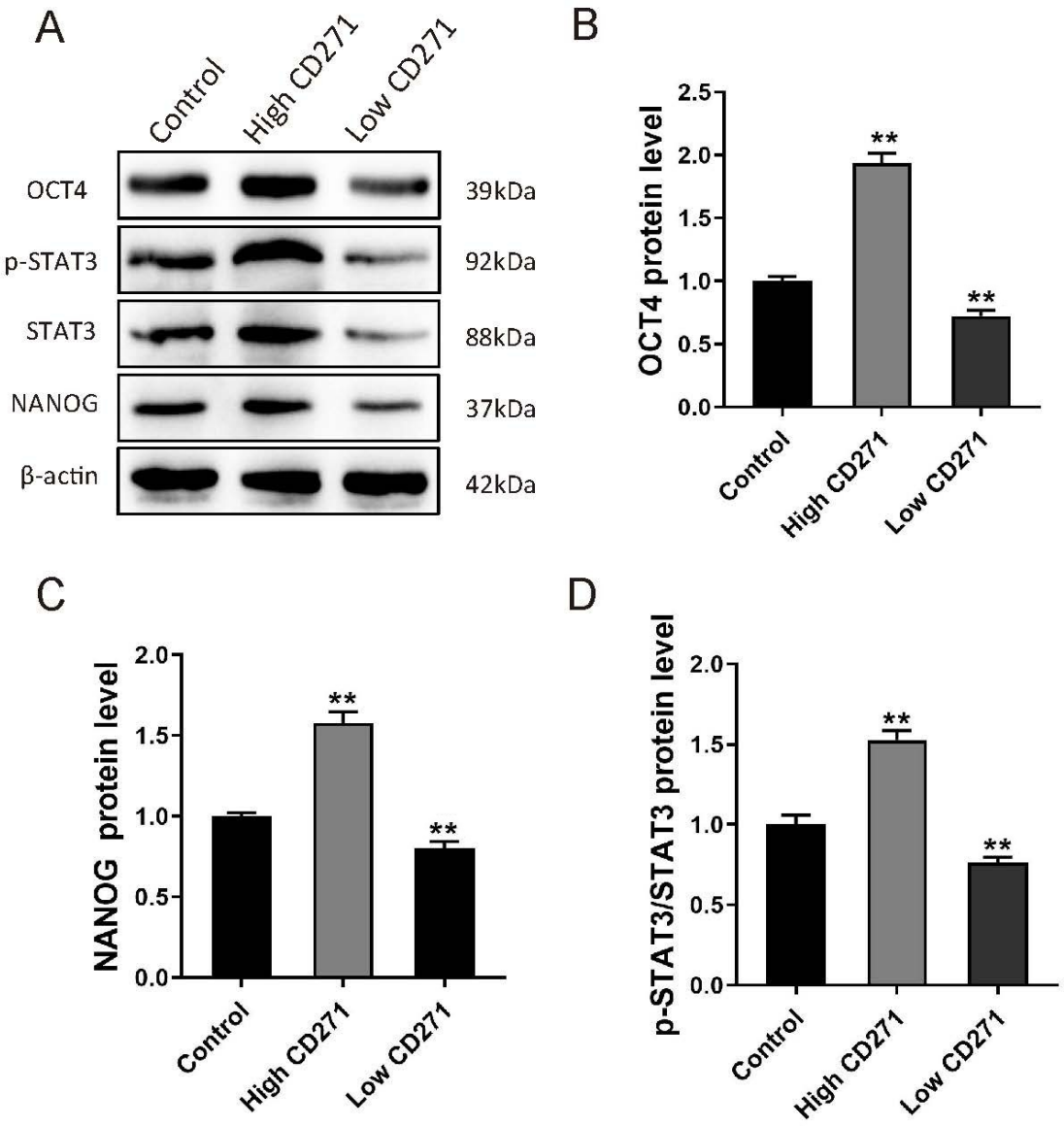


**Fig. 5. The protein level of OCT4, NANOG, p-STAT3, and STAT3 in Hep-2 cells with CD271 high/low expression.** (A) Western blot assay was used to detect the protein level of OCT4, p-STAT3, STAT3, and NANOG. (B) Relative protein expression of OCT4. (C) Relative protein expression of NANOG. (D) Relative protein expression of p-STAT3/STAT3. $^{**}p < 0.01$. STAT3, signal transducer and activator of transcription 3; OCT4, Octamer-binding transcription factor 4; NANOG, Nanog Homeobox. n = 3.

tional roles of CD271 vary among different tumor types; for instance, in gastric cancer, prostate cancer, and bladder cancer, CD271 often exhibits potential tumor-suppressive functions, while in melanoma, malignant glioma, and breast cancer, it may promote tumor progression [17]. IHC staining conducted in this study revealed widespread CD271 positivity in most laryngeal squamous cell carcinoma tissues. In well-differentiated squamous carcinoma tissues, CD271-positive cells were predominantly situated in basal layers around cancer nests and infiltration margins connecting with the mesenchyme [10]. Immunocytochemical staining on laryngocarcinoma Hep-2 cells further demonstrated that a subset of Hep2 cells exhibited strong positivity for CD271. Concurrently, observations revealed that many Hep2 cells in the DNA synthesis phase, cell division stage, and anaphase exhibited robust CD271 immunostaining.

A prior study identified a correlation between high expression of NGF and TrkA with laryngeal cancer metastasis and malignant progression. Therefore, the proliferation-promoting function of CD271 in laryngocarcinoma is likely closely associated with NGF and TrkA expression [18]. The regulatory mechanism is intricate, particularly considering the multifaceted biological functions of the CD271 receptor [19]. These receptors transmit intracellular signals through various canonical pathways, including the mitogen-activated protein kinase (MAPK)

and phosphatidylinositol-3 kinase (PI3K)/Protein Kinase B (AKT) signaling pathways [20–22]. While previous CD271 studies have predominantly focused on the nervous system [21,23], numerous investigations have confirmed its role in promoting neuronal survival, proliferation, differentiation, apoptosis induction, and participation in cell cycle regulation [9]. In Hep-2 cells, those with high CD271 expression exhibited stronger proliferation ability and tumor stem cell sphere formation, whereas cells with low CD271 expression demonstrated weaker capabilities. Immunocytochemical staining revealed a small subset of Hep-2 cells expressing CD271 at high levels, with some of these strongly positive cells exhibiting exuberant proliferation. These findings underscore the essential role of CD271 in the survival and proliferation of Hep-2 cells.

CSCs, also known as TICs possess stem cell-like characteristics [24]. They exhibit the capacity for self-renewal, robust proliferation, and differentiation into various cell types, ultimately contributing to tumorigenesis [25]. TICs directly influence tumor occurrence and development, closely impacting resistance, recurrence, and patient survival time. They represent significant targets for tumor treatment, garnering widespread attention. Understanding the operational mechanisms of TICs and identifying intervention methods holds clinical application value [26]. In our study, we observed a close association between the expression of CD271 and the stemness of laryngeal cancer cells. CD133 expression was elevated in CD271 high-expression Hep-2 cells compared to CD271 low-expression counterparts. CD271, a pivotal factor in TICs, exhibited a positive correlation with the expression of tumor stemness markers, tumor sphere formation ability, and tumor drug resistance.

As a member of the TNFR family, it is understandable that CD271 possesses tumor inhibition functions [27]. A study on oral squamous cell carcinoma revealed that up-regulation of CD271 expression and alterations in its distribution patterns could serve as indicators for recurrence and poor prognosis post-treatment [28]. Okumura and colleagues [29] reported that CD271 supported cell survival and growth in esophageal squamous cell carcinoma. Another study demonstrated that, upon combination with its ligand NGF, CD271 could inhibit apoptosis in breast cancer through the activation of the NF-$\kappa$B pathway [30]. However, the precise mechanism of CD271 in tumors remains unclear. Currently, the survival-promoting mechanism mediated by CD271 has been identified in esophageal squamous cell carcinoma [31]. Our findings indicate that CD271 high expression increased the protein expression of NANOG, OCT4, STAT3, and p-STAT3 in Hep-2 cells. Both total protein levels and phosphorylation of STAT3 were increased in CD271 high-expression Hep-2 cells, which may indicate that both STAT3 expression and activation were promoted. This may be due to activation of upstream signaling pathways, leading to increased synthesis of STAT3 protein, as well as enhanced status of its phosphorylation. Changes in total protein levels of STAT3 may be related to protein synthesis and degradation processes, while changes in phosphorylation may be related to kinase and phosphatase activities. Prior to being considered a potential target for therapies, further research is needed to elucidate the regulatory mechanism of CD271 and its associated signaling pathways.

## Conclusions

CD271 was found to be over-expressed in a small subset of human laryngeal carcinoma epithelial Hep-2 cells, indicative of high proliferation energy or self-renewal capability. Our results imply that CD271 is essential for the survival and proliferation of Hep-2 cells, potentially serving as a marker for TICs in Hep-2. Further investigations are warranted to delve into the regulatory mechanisms of CD271 and its associated signaling pathways. Overall, CD271 promotes CSCs through the STAT3 signaling pathway in laryngocarcinoma.

## Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

## Author Contributions

YPS: Conceptualization; formal analysis; funding acquisition; investigation; methodology; validation. XL: Writing original draft; investigation; and formal analysis. CZX: Resources; investigation; methodology. JHZ: Validation and Visualization; Writing—review and editing. FG: Cytological study and validation. RFZ: Validation; Editing. DL: Investigation; data curation. BHZ: Morphology investigation. XML: Resources; supervision; conceptualization. All authors were involved in the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

In accordance with the principles and requirements of the Helsinki Declaration, the study protocol was approved by the review board of the Ethics Committee of the Eye Ear Nose and Throat Hospital, Fudan University (approval number: 2021039). Informed consent: Paraffin-embedded human laryngeal squamous cell carcinoma tissues were obtained from the Bethune International Peace Hospital with written informed consent. All animals were kept according to the Guidelines on the Use of Animals in Research of the Institutional Animal Care and the National Animal Welfare

Law and are approved by the responsible government authority (Experimental Animal Ethics Committee of Bethune International Peace Hospital, approval number: 2017-KY-13). All efforts were made to minimize the suffering of the animals.

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537194.46.

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