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# Expression and clinical significance of YAP in laryngeal squamous cell carcinoma patients

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**Abstract.** The aim of the present study was to investigate the expression levels of Yes-associated protein (YAP) in different grades of laryngeal squamous cell carcinoma (LSCC) tissues and vocal cord polyps tissues, and to investigate any correlations with clinical factors. The expression of YAP in 128 cases of LSCC and 10 cases of vocal cord polyps tissues was tested using immunohistochemistry. YAP was primarily present in the nucleus of LSCC and controls, whereas phosphorylated YAP expression was present in the cytoplasm. The results indicated that YAP expression was upregulated in LSCC samples compared with vocal cord polyps tissues. YAP expression was positively correlated with the malignant degree of LSCC ($P<0.01$) and a high level of YAP expression in LSCC tissues was correlated with pathological type, lymphatic metastasis and clinical stage. The present study provided evidence for the expression and localization of YAP in LSCC and vocal cord polyps tissues. Thus, YAP may be involved in the occurrence and development of LSCC as an oncogene.

## Introduction

Laryngeal carcinoma is a common malignant tumor in otolaryngology that seriously endangers physical and mental health (1). The incidence of laryngeal malignant tumors is ~1-5% of all whole body malignant tumors and is ranked third of all otolaryngology malignant tumors. Laryngeal squamous cell carcinoma (LSCC) accounts for 95% of laryngeal carcinoma and tends to occur in middle-aged and older males (2). Due to the early presence of hoarseness (3), glottic carcinoma can be detected timely. However, it brings challenges for the diagnosis of LSCC owing to the unapparent symptoms in the early stage of supraglottic and subglottic carcinomas (4). Early diagnosis and treatment of LSCC are critical in improving the 5-year survival rate and quality of life of patients (5-7).

The Hippo pathway was originally observed to be a key pathway that is involved in the regulation of the size of organs of *Drosophila melanogaster* by regulating cell proliferation and apoptosis (8). Previous studies on the regulation of the development and size of mammals (9-11) indicated that the Hippo pathway was quite conservative. It was also demonstrated that abnormal functions of Hippo pathway were closely associated with the occurrence, development and prognosis of tumors (12,13), which has become a popular subject of cancer research.

The aim of the present study was to investigate the expression and localization of YAP in LSCC and vocal cord polyps tissues as well as the clinical significance thereof.

## Materials and methods

*Sample collection.* Approval for the present study was obtained from the Human Research Ethics Committee of Lihuili Hospital, Ningbo, China. Prior to sample collection, the patients and their family members were informed with regard to the process of the study and the possible risks. Tissues were collected from 128 patients with LSCC and 10 patients with vocal cord polyps between January 2009 and January 2013 at the Ningbo University Affiliated Lihuili Hospital. All patients signed informed consent. Patients were examined and diagnosed by two experienced pathologists. LSCC was classified using the American Joint Committee on Cancer 2002 (AJCC 2012).

*Primary reagents.* Primary reagents included rabbit anti-human YAP polyclonal antibody (Abcam, Cambridge, UK), rabbit anti-human p-YAP polyclonal antibody (Abcam), Envision™ Kit (Dako, Glostrup, Denmark), citrate antigen repair solution (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., China), 3% hydrogen peroxide (Dako), sheep serum (Beijing Boshide Technology Co., Beijing, China), a diaminobenzidine tetrahydrochloride (DAB) kit (Dako), hematoxylin (Sigma, Shanghai, China), phosphate buffered-saline (PBS) (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd.), xylene (Anhui Ante Biochemistry Co., Ltd., China), absolute ethyl alcohol (Anhui Ante Biochemistry Co., Ltd.), hydrochloric acid (Anhui Ante Biochemistry Co., Ltd.) and neutral quick-drying

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*Key words:* larynx squamous cell carcinoma, yes-associated protein

glue (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd.).

*Primary instruments*. Instruments used included an RM2135 paraffin slicing machine (Leica, Munich, Germany), HI1210 water bath-slide drier (Leica), GNP-9050 water-prevented constant temperature incubator (Shanghai Jinghong Laboratory Equipment Co., Ltd., Shanghai, China), BX41 optical microscope (OLYMPUS, Tokyo, Japan), microscopic imaging system (OLYMPUS), Milli-Q Reference pure water filter (Millipore, Billerica, MA, USA), pressure cooker (ASD Company, Conover, NC, USA) and BCD-268H refrigerator (Haier Company, Shandong, China).

*Immunohistochemistry*. Paraffin blocks of normal tissues surrounding carcinoma and laryngeal squamous cell carcinoma tissues, which were verified by hemotoxylin and eosin staining, were selected and cut into 2 $\mu$m sections. The slices were mounted on cationic glass slides for 4 h baking at 60˚C, followed by dewaxing and hydration. Antigen repair was subsequently performed. Citrate buffer (pH 6.0) in a concentration of 1 mol/l was added into a pressure cooker. After the pressure valve was closed, the pressure cooker was heated using an electric stove until air gush and the heating stopped after 2 min boiling. The glass slides were removed following natural cooling at room temperature, rinsed using distilled water and finally washed by PBS three times for 2 min each time. The slides were processed by freshly prepared 3% $H_2O_2$ for 15 min at room temperature to inactivate endogenous peroxidase. Following three washes with PBS (2 min each time), the slices were incubated at room temperature following the addition of 1% protein liquid. After 10 min, the slices were washed with PBS and antibody binding reaction was subsequently performed. Rabbit anti-human YAP polyclonal antibody and rabbit anti-human p-YAP polyclonal antibody (1:200, diluted with phosphate buffer solution (pH 7.4) (ZLI-9062, Beijing Zhongshan Jinqiao Biotech Co., Ltd., Beijing, China)) were added to cover all the specimens. The slices were placed in a wet box for 18 h incubation at 4˚C and subsequently rinsed with PBS three times for 2 min each time. Envision™ reagent (general type) was added to cover all the specimens, followed by 20-min incubation at 37˚C and rinsing three times with PBS (2 min each time). One or two drops of DAB were added to each slice for coloration. After 2 min, the staining was observed under a microscope (Olympus BX41, Olympus Corp, Japan), followed by full washing by tap water. Following redye with hematoxylin, the slices were fully washed with water to repeat staining for 1 min, and then were fully washed in water. After dehydration, the slices were mounted by neural drying rubber and observed under a microscope.

*Determination of immunohistochemical results*. A positive expression of YAP in carcinoma tissues presented as brown yellow or brown granules in the tumor cytoplasm or cell membrane. The tumor solid area was selected under a x100 lens and 5 views were selected under x400 lenses. The result was scored according to the staining intensity: 0, negative; 1, weak positive; 2, moderate positive; and 3, strong positive. The number of cells with positive staining results was graded as: ≤10%, i.e., 0, negative; 11-30%, i.e., weak positive, for 1 point; 31-50%, moderate positive, for 2 points; and > 50%, i.e., strong positive, for 3 points. When the sum of these two scores was ≥3 points, the expression of YAP was determined as positive.

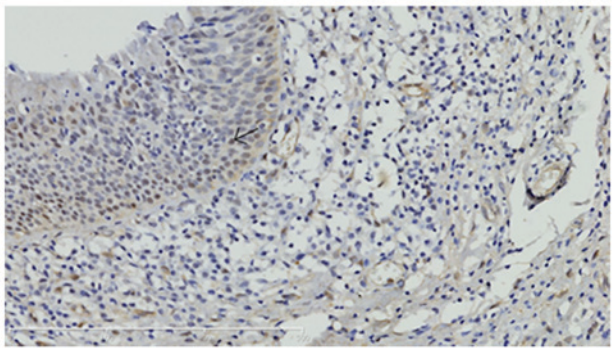

Figure 1. Weak positive YAP expression in highly differentiated squamous-cell carcinoma tissue (magnification, x10). Staining, hemotoxylin and eosin; YAP, yes-associated protein.

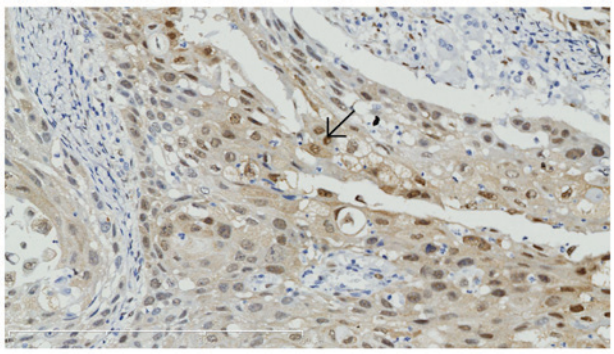

Figure 2. YAP expression sites within tumor cell nucleus (magnification, x40). Staining, hemotoxylin and eosin (immunohistochemical Envision method). YAP, yes-associated protein.

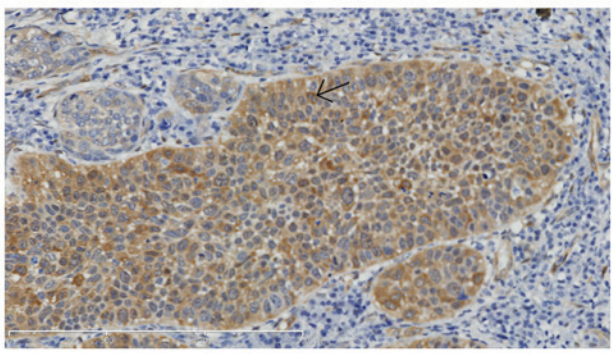

Figure 3. p-YAP expression sites within tumor cell cytoplasm (magnification, x10). Staining, hemotoxylin and eosin; p-YAP, phosphorylated yes-associated protein.

*Statistical analysis*. SPSS ver. 18.0 software (Chicago, IL, USA) was used for statistical analysis. The correlation between a positive expression rate of YAP and pathological stages was analyzed using the $\chi^2$ test. Differences were considered statistically significant when $P<0.01$.

Table I. Correlations of YAP and p-YAP with different clinical factors.

| | YAP | | | | | P-YAP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | - | + | ++ | +++ | P-value | - | + | ++ | +++ | P-value |
| Age (years) | | | | | 0.746 | | | | | 0.629 |
| <59 | 1 | 31 | 27 | 7 | | 3 | 29 | 24 | 10 | |
| >60 | 3 | 27 | 25 | 7 | | 5 | 22 | 27 | 8 | |
| Clinical stage | | | | | <0.001[a] | | | | | <0.001[a] |
| Tis | 4 | 5 | 0 | 0 | | 7 | 2 | 0 | 0 | |
| I | 21 | 11 | 1 | 0 | | 1 | 22 | 9 | 1 | |
| II | 0 | 11 | 10 | 0 | | 0 | 11 | 10 | 0 | |
| III | 0 | 14 | 16 | 6 | | 0 | 10 | 19 | 7 | |
| IV | 0 | 7 | 15 | 7 | | 0 | 6 | 13 | 10 | |
| Lymphatic metastasis | | | | | <0.001[a] | | | | | <0.001[a] |
| Yes | 0 | 11 | 23 | 12 | | 0 | 8 | 22 | 16 | |
| No | 4 | 47 | 29 | 2 | | 8 | 43 | 29 | 2 | |
| Smoking history | | | | | 0.279 | | | | | 0.56 |
| Yes | 2 | 43 | 38 | 13 | | 5 | 36 | 41 | 14 | |
| No | 2 | 15 | 14 | 1 | | 3 | 15 | 10 | 4 | |
| Gender | | | | | 0.71 | | | | | 0.003 |
| Male | 3 | 56 | 51 | 14 | | 6 | 50 | 50 | 18 | |
| Female | 1 | 2 | 1 | 0 | | 2 | 1 | 1 | 0 | |
| Pathological type | | | | | <0.001[a] | | | | | <0.001[a] |
| Highly differentiated | 4 | 52 | 11 | 0 | | 8 | 45 | 14 | 0 | |
| Moderately differentiated | 0 | 6 | 38 | 8 | | 0 | 6 | 34 | 12 | |
| Lowly differentiated | 0 | 0 | 3 | 6 | | 0 | 0 | 3 | 6 | |

[a]P<0.001 indicates a statistically significant association. YAP, yes-associated protein; Tis, tumor *in situ*.

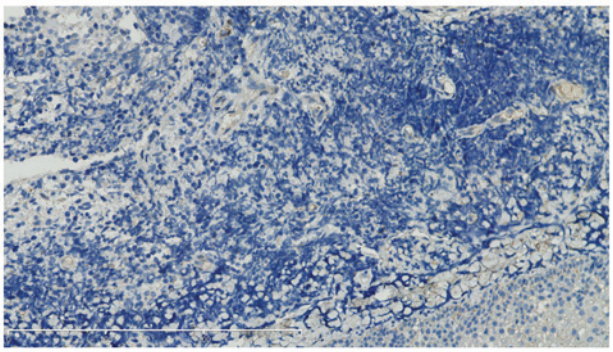

Figure 4. Negative YAP expression in vocal cord polyps (magnification, x10). Staining, hemotoxylin and eosin; YAP, yes-associated protein.

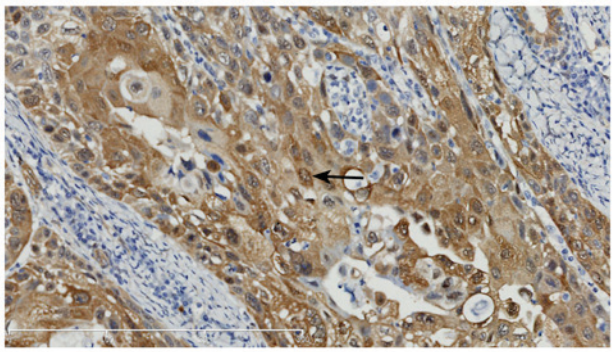

Figure 5. Strong positive YAP expression in poorly differentiated squamous-cell carcinoma tissue (magnification, x40). Staining, hemotoxylin and eosin. YAP, yes-associated protein.

## Results

*Expression level of YAP in LSCC and vocal cord polyps.* YAP and phosphorylated YAP (p-YAP) were expressed in the nucleus and cytoplasm of LSCC cells, respectively, whereas YAP was negatively expressed in vocal cord polyps (Figs. 1-5). In addition, the intensity of YAP expression varied in LSCC with different histological differentiation degrees and poorly differentiated LSCC exhibited higher expression of YAP.

*Correlation between YAP expression and clinicopathological characteristics of LSCC patients.* The correlation between YAP expression in LSCC and the clinicopathological characteristics was analyzed using the $\chi^2$ test. Table I demonstrates that the expression of YAP was significantly associated with tumor-node-metastasis stage (P<0.001), lymph node metastasis (P<0.001) and pathological differentiation (P<0.001). However, there was no correlation with age, smoking history or gender.

## Discussion

YAP determines the size of organs by regulating cell proliferation and apoptosis. It is a transcriptional co-activator that monitors cell behavior in the nucleus and is assisted by transcription factors. The signaling pathway of the Hippo kinase-mediated cascade is important in the phosphorylation of YAP (14). It has been suggested that YAP may be a tumor suppressor and an oncogene (15,16). For example, levels of YAP mRNA in pancreatic cancer tissues were 2.5- and 1.3-fold higher than those observed in normal pancreas tissues and chronic pancreatitis tissues, respectively. Similar results have been observed in gastric and liver cancer (17,18). However, the role of YAP in the occurrence of tumors as an oncogene has been questioned (19-21). The expression of YAP was observed to be downregulated or even lost, YAP-deficit breast cancer cells demonstrated stronger potentials of invasion and metastasis, and results from *in vivo* studies indicated that tumors occurred early and grew rapidly in YAP knockout nude mice (22,23). However, the role of YAP in LSCC has yet to be elucidated.

In the present study, immunohistochemistry was used to detect the expression level of YAP in vocal cord polyps and laryngeal squamous cell carcinoma tissues. The results demonstrated that YAP was overexpressed in LSCC and positively correlated with the histological differentiation degree of tumors. The analysis of the clinicopathological characteristics also demonstrated that YAP expression was correlated with TNM stage, lymph node metastasis and pathologic differentiation. These results suggest that YAP is an independent prognostic biomarker of LSCC as an oncogene. The results of the current study are consistent with a number of previous studies reporting that YAP expression is linked with tumor evolution and progression (24). Thus, YAP is a potential treatment target for LSCC (25).

In conclusion, the present study has provided evidence for the expression and localization of YAP in LSCC and vocal cord polyps tissues. Additionally, YAP may be involved in the occurrence and development of LSCC as an oncogene. However, further studies are required to determine whether targeting YAP inhibits the organ size of patients with tumors.

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