

# Integrin-linked kinase is overexpressed in laryngeal squamous cell carcinoma and correlates with tumor proliferation, migration and invasion

P.-A. WU<sup>1,4</sup>, L.-L. XIE<sup>2</sup>, D.-Y. ZHAO<sup>3</sup>, S.-S. LI<sup>4</sup>, Q.-L. TANG<sup>4</sup>, S.-H. WANG<sup>4</sup>, X.-M. YANG<sup>4</sup>

<sup>1</sup>Department of Otolaryngology, Head and Neck Surgery, the University of Hong Kong-Shenzhen Hospital, Shenzhen, Guangdong, P.R. China

<sup>2</sup>Department of Clinical Microbiology and Infection Control, the University of Hong Kong-Shenzhen Hospital, Shenzhen, Guangdong, P.R. China

<sup>3</sup>Central Laboratory, the University of Hong Kong-Shenzhen Hospital, Shenzhen, Guangdong, P.R. China

<sup>4</sup>Department of Otolaryngology, Head and Neck Surgery, the Second Xiangya Hospital, Central South University, Changsha, Hunan, P.R. China

**Abstract. – OBJECTIVE:** To investigate the role of integrin-linked kinase (ILK) in invasion and metastasis of the laryngeal squamous cell carcinomas (LSCC) and to evaluate the effects of antisense oligonucleotide sequence (ASONS) targeting the ILK gene on the proliferation, epithelial-mesenchymal transition (EMT), migration and invasion of LSCC.

**PATIENTS AND METHODS:** 116 patients who had previously undergone complete resection of the tumor for LSCC were studied retrospectively. The ILK expression level in tumor tissues and adjacent normal tissues were determined by immunohistochemistry. The changes of ILK expression from each group were assessed and correlated to the clinical parameters of the patients. Secondly, ILK antisense oligonucleotide (ILK-ASONS) was used to silence the ILK gene of LSCC cell from Hep-2 cell line. The expression of ILK, epithelial marker E-cadherin and mesenchymal marker Vimentin were evaluated by Western blotting. The proliferation of cells after transfection was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The apoptosis was detected by flow cytometry. The migration and invasion activity of Hep-2 cells was detected by Matrigel invasion and cell migration assays.

**RESULTS:** The expression of the ILK protein was significantly associated with tumor differentiation ( $p=0.046$ ), lymph node metastasis ( $p=0.020$ ) and pTNM stage ( $p=0.019$ ). ILK ASONS-transfected cells showed a significant decrease in cell proliferation, cell migration and invasive activity compared to mock-transfected cells. ILK ASONS-transfected cells increased the expression of E-cadherin, whereas the expression of ILK and Vimentin decreased, compared with mock-transfected cells.

**CONCLUSIONS:** The expression of ILK was significantly correlated with differentiation and metastasis of the laryngeal carcinomas. The inhibition of the ILK gene could downregulate the proliferation, migration and invasion of Hep-2 cells. These findings suggest that the ILK gene could be a potential target for the treatment of laryngeal cancer.

## Key Words

Integrin-linked kinase (ILK), Laryngeal carcinoma, Antisense oligonucleotides, Epithelial-to-mesenchymal transformation (EMT).

## Introduction

Laryngeal squamous cell carcinoma (LSCC), one of the most common malignancies of the head and neck region which causes morbidity and mortality worldwide, accounts for approximately 5% of all human cancers. Over the past few decades, diagnosis and therapy of laryngeal carcinoma have substantially improved, but the long-term survival has not increased significantly. The five-year survival is approximately 63% in the United States<sup>1</sup>. Lymph node metastasis is the most important prognostic factor in these patients<sup>2</sup>. Currently, surgery is the most widely used treatment strategy for these tumors, but new treatment methods that provide more effective results are highly desirable due to reduced side effects. Novel targets that can serve as the foundation for biologically based therapeutic strategies

are needed to develop new treatment modalities.

A promising target is integrin-linked kinase (ILK), which was discovered as a  $\beta 1$ -integrin subunit cytoplasmic domain interactor in 1996, associated with the growth and survival of anchorage-dependent cells, cell cycle progression, epithelial-mesenchymal transition (EMT), cell motility and contraction, vascular development, tumor angiogenesis, invasion and migration<sup>3</sup>. The overexpression of ILK has been shown to induce the formation of tumors in nude mice<sup>4-6</sup>. ILK small-molecule inhibitors have been identified to inhibit tumor growth, invasion and angiogenesis<sup>7,8</sup>.

EMT is a critical key process in embryonic development and is assumed to participate in the invasion and metastasis of carcinomas. EMT is a mechanism by which epithelial cells, generated in a particular region, can be transformed into a mesenchymal phenotype, dissociate from the epithelium and migrate to a secondary site. During EMT, epithelial genes such as E-cadherin are downregulated, whilst mesenchymal genes such as Vimentin are upregulated<sup>9,10</sup>.

In a previous study<sup>11</sup>, we investigated the expression of ILK in human laryngeal squamous cell carcinoma specimens and found that the increased expression of ILK is associated with lymph node metastases and a poor patient survival rate. To further investigate the role of ILK in laryngeal squamous cell carcinomas proliferation, apoptosis, epithelial-mesenchymal transition and tumor metastasis, we knocked down the ILK expression by antisense oligonucleotide sequence (ASONS) in the Hep-2 cell line and our data suggested that ILK acts as an oncogene in laryngeal squamous cell carcinoma and may provide a novel clue in diagnosis and therapy in laryngeal cancer.

## Materials and Methods

### *Tissue Specimens and Immunohistochemistry*

A total of 116 human laryngeal cancer specimens and adjacent non-cancerous tissue (1 cm distance from the primary tumor at least) were collected from the Department of Otolaryngology-Head and Neck Surgery, the Second Xiangya Hospital of Central South University. Patients involved in this study signed the informed consent. The entire study was approved by the Ethics Committee of the hospital. Histologic grading

and TNM classification were staged according to the 6<sup>th</sup> edition of cancer staging manual of the International Union Against Cancer (UICC, 2002). Immunohistochemical staining of tumor specimens and statistics were done as described previously<sup>11</sup>. The results of immunohistochemical staining were scored by two experienced pathologists independently and according to the percentage of immunoreactive cells (quantity score) and staining intensity (staining intensity score). The pathologists were blinded to each other. Briefly, five high-powered fields under the microscope were randomly chosen and one hundred cells in each field were counted. The staining scores (IHS) were calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score). For the percentage of staining, score 0 indicated no staining; score 1, 1-10% of cells stained; score 2, 11-50%; score 3, 51-80%; score 4, 81-100%. Staining intensity scores were as follows: score 0, no staining; score 1, light yellow (weak); score 2, yellow (moderate); and score 3, deep yellow (strong). We defined the IHC staining score as the intensity score multiplied by the percentage score. Patients were classified into two groups according to the staining score of tumor tissues as follows: samples with scores (0-6) of tumor stained as negative staining and samples with scores (7-12) of tumor stained were considered as positive<sup>11</sup>.

### *Culture of Laryngeal Cancer Cells (Hep-2)*

Hep-2 human laryngeal squamous cell carcinoma cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and containing 100 units/ml of penicillin and 100 units/ml of streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### *ILK ASONS Design and Transfection*

According to some studies<sup>8</sup>, the ASONS-targeting ILK gene and the control sequences (sense and mismatched oligonucleotide sequences), which comprised a 20-mer with a fluorescein isothiocyanate (FITC) tag at the 5' end, were synthesized by Integrated DNA Technologies (Coralville, IA, USA). To reduce intracellular cleavage by degradative enzymes and increase stability, the oligonucleotides

were phosphorothioate oligodeoxynucleotides with 2'-O-methyl (placing the 12 2'-O-methyl groups in two equal blocks at both ends to form a gap-mer) incorporated to enhance their affinity for RNA sequences and their resistance to degradation by nucleases. The antisense sequence was 5'-AmUmGmUmCmGTC-CATAGCmAmGmCmGmUmC-3' (20-mer), the mismatched sequence was 5'-mGmUmAmGmCmCGCTAGTACmUmCmGmAmUmC-3' (20-mer). All oligonucleotides were dissolved in double-distilled water and stored in aliquots at -20°C. To evaluate the transfection efficiency, antisense and mismatched oligonucleotides (final concentration, 200 nM, respectively) were mixed with 1.5 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), incubated for 20 min and transfected into 80% confluent Hep-2 cells. Hep-2 cells were cultured with oligonucleotides in serum-free medium for 6 h at 37°C and followed by the addition of fetal bovine serum (FBS) (final concentration 10%). After transfection of oligonucleotides, cells were added to 6-well plates (5×10<sup>5</sup> cells per well) and the transfection efficiency was evaluated using by fluorescence microscope. Hep-2 cells treated with Lipofectamine 2000 alone were used as normal controls.

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

Cell viability after transfection of oligonucleotide was assessed by MTT assay. Hep-2 cells seeded into 96-well plates at a density of 4×10<sup>3</sup> cells per well and incubated for 24 hours in RPMI-1640 medium supplemented with 10% fetal bovine serum. After transfections of antisense (ASONS) and mismatched (ASONS-C) oligonucleotides (final concentration, 200 nM, respectively) for 24, 48, 72 h and 96 h respectively; 20 µl of sterile MTT dye (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for an additional 4 h at 37°C. Then, 150 µl of dimethyl sulphoxide was added to each well and thoroughly mixed for 10 min. Cells treated with Lipofectamine 2000 alone were used as negative controls (Normal). The optical absorbance was measured at 490 nm on an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA). The relative cell viability was calculated by comparison with the control group cells. For each experimental condition, the assay was performed in triplicate.

#### **Cell Apoptosis Detection by Flow Cytometry**

After 48 h of transfection with 200 nM ASONS or mismatched oligonucleotides, cells were washed twice with cold Phosphate-Buffered Saline (PBS) and resuspended in binding buffer at a concentration of 1×10<sup>6</sup> cells/ml. Cell suspensions were fixed with 70% ethanol, washed with the APO-DIRECT Wash Buffer (BD Biosciences, Franklin Lakes, NJ, USA). Cell apoptosis was assessed by Annexin V-FITC/propidium iodide (PI) staining with the flow cytometric apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by using the FACScan<sup>TM</sup> (BD Biosciences, Franklin Lakes, NJ, USA). Approximately, 10,000 cells were obtained per sample. Hep-2 cells treated with Lipofectamine 2000 alone were used as internal controls and the experiments were repeated in triplicate.

#### **Antibodies and Western Blotting**

Western blotting was performed using standard procedures. After transfection with 200 nM ASONS or mismatch oligonucleotides for 48 h, cells were harvested and washed with ice-cold PBS. Whole-cell extracts were prepared using cell lysis buffer (50 mmol Tris, pH 8.0, 1% Triton X-100 and 100 g/ml phenylmethylsulphonyl fluoride) and cleared by centrifugation at 14,000×g at 4°C for 15 min. Equal amounts of protein were loaded onto 6% polyacrylamide gels for SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After the non-specific sites were blocked, anti-ILK (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E-cadherin (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-vimentin (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin antibody antibodies (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect the respective proteins. After incubation, the sections were rinsed with PBS and incubated with the secondary biotinylated goat anti-rabbit IgG (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) detection was performed according to the manufacturer's instructions. Blots were visualized using an image analyzer and the protein expression was quantified using an ImageQuant densitometric scanner (Amersham Biosciences, Amersham, Buckinghamshire, UK).

### **Matrigel Invasion Assay and Cell Migration Assay**

Cell migration and invasion assays were performed using a 24-well transwell unit (8  $\mu$ m pore size) containing Polyvinylpyrrolidone-free polycarbonate filters that were coated (invasion assay) or uncoated (migration assay) with 1 mg/ml of BD Matrigel Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ, USA). Cells ( $8 \times 10^4$  cells/ml) were suspended in RPMI-1640 containing 10% FBS and seeded into the upper chambers of Matrigel-coated transwell plates. The cells were placed in the upper compartment of the migration chamber and attached for 8 h before incubation in FBS-free medium for 24 h at 37°C in a 5% CO<sub>2</sub> atmosphere. The lower compartment of the migration chamber contained RPMI-1640 with 10% FBS. Non-invading cells remaining on the upper surface of the filter were removed and the cells that appeared on the lower surface of the filter were fixed with 95% ethanol for 10 min, stained with hematoxylin and eosin (HE), and counted in five random high-power fields (HPF) under an inverted microscope (Nikon Eclipse, Tokyo, Japan). Under each experimental condition, assays were performed a minimum of three times. The number of invading cells in the three Boyden chambers was counted and the mean was calculated.

### **Statistical Analysis**

The association between the ILK expression and clinicopathological parameters was statistically evaluated by using Fisher's exact test or chi-square test. The results are presented as the mean  $\pm$  standard deviation of at least three independent experiments performed on separate days. Differences between the means of the individual groups were assessed by one-way ANOVA with SNK-*q* tests. The SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistics and  $p < 0.05$  was considered to be statistically significant.

## **Results**

### **Immunohistochemical Staining**

Immunohistochemical staining for ILK was performed in 116 tumors and adjacent non-neoplastic laryngeal tissue. A distinct brown or brown-yellow cytoplasmic and/or the nucleus staining of cancer cell was considered as a

positive expression for ILK and was observed the majority of the LSCC cases (76/116, 65.5%). In contrast, a few of adjacent non-tumor laryngeal tissues were also immunolabeled for ILK (24/116, 20.7%) with a significantly lower expression rate compared with that in primary tumors ( $p < 0.001$ ; Figure 1A, B, C and D). This result showed that the ILK was over-expressed in LSCC compared to tumor-adjacent normal tissues suggesting that ILK is up-regulated in LSCC cells.

### **ILK Expression Levels in Groups of Clinic Pathological Parameters**

As shown in Table I, there was no statistically significant correlation between the ILK expression status and clinical factors of age, gender, tumor site and tumor size. However, the expression of the ILK protein was much higher ( $p = 0.020$ ) in the primary tumors associated with lymph node metastasis (pN<sub>+</sub>, 49/65) than in those without (pN<sub>0</sub>, 27/51). The expression of the ILK protein in the advanced tumors (stage III-IV, 53/72) was significantly higher ( $p = 0.019$ ) than that in early tumors (stage I-II, 23/44). In addition, a significant difference ( $p = 0.046$ ) was found between the degrees of differentiation with lower expression of the ILK protein in the well-differentiated tumors (32/58) and higher expression in moderately ( $n = 29/40$ ) or poorly differentiated tumors ( $n = 15/18$ ). Thus, tumor differentiation was inversely correlated with the expression level of ILK.

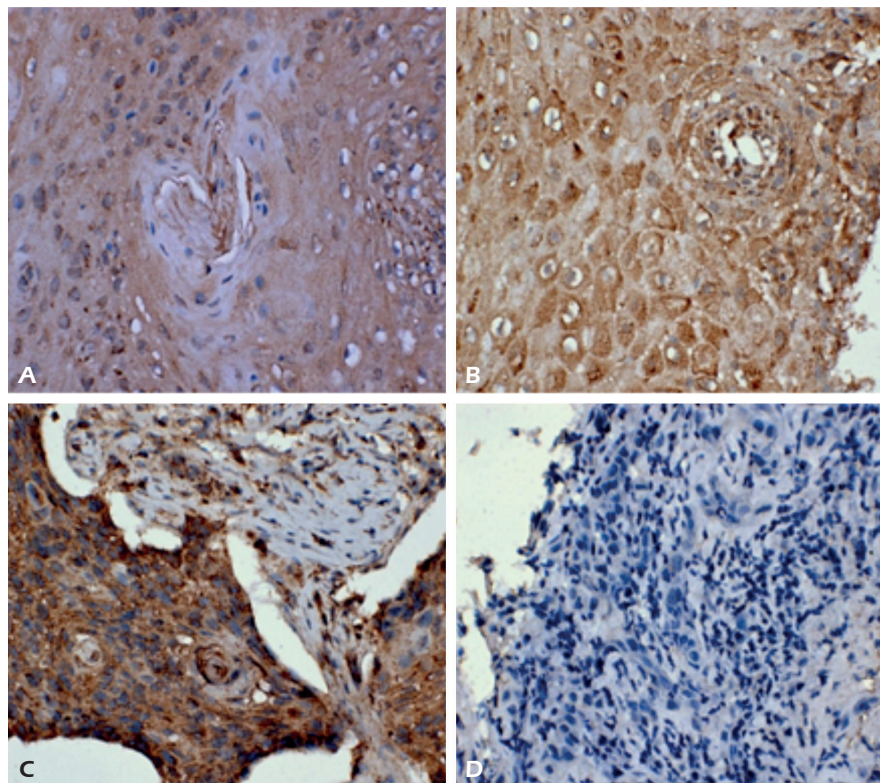
### **Evaluation of Transfection Efficacy of ILK ASONS**

To detect whether the oligonucleotide sequences could be successfully transfected into Hep-2 cells, a fluorescent decoration (FITC) was used as a marker at the 5' end of antisense or control sequences to assess transfection efficiency. The transfection efficacy was approximately 85% (Figure 2).

### **Inhibitory Effects of ASONS on Hep-2 Cell Viability**

As shown in Figure 3, the viability of Hep-2 cells after treatment with ASONS was significantly decreased at each time point (48, 72 and 96 h, respectively). However, survival rates of mismatched sequences (ASONS-C) groups did not show any alteration in the time course. These curves showed that ASONS decreased the viability of Hep-2 cells.

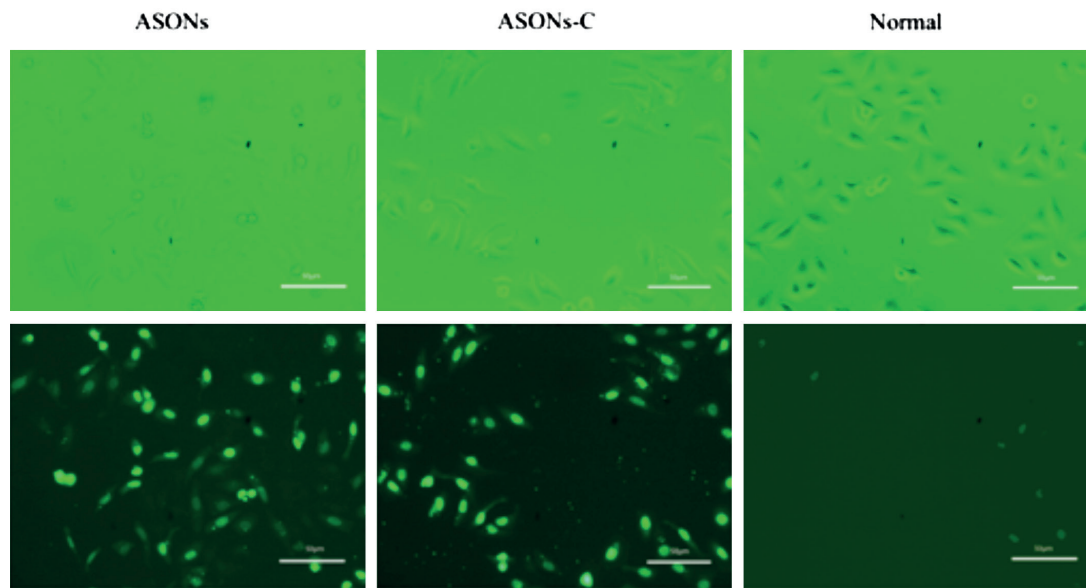




**Figure 1.** Immunohistochemical studies of ILK in tumor tissue of LSCC. **A**, Light Yellow Granular staining in cytoplasmatic expression pattern indicated weak positive expression of ILK. **B**, Yellow Granular staining in cytoplasmatic expression pattern indicated moderate positive expression of ILK. **C**, Deep Yellow Granular staining in cytoplasmatic and nuclear expression pattern indicated strong positive expression of ILK. **D**, No staining in cytoplasmatic and nuclear expression pattern indicated negative staining of ILK. Original magnification: 200 × in **A**, **B**, **C** and **D**.

**Table I.** Correlations of ILK expression and clinicopathological parameter (\* $p < 0.05$ ).

| Clinicopathologic variables |              | Number of patient | ILK protein expression |          | $p$ ( $X^2$ )  |
|-----------------------------|--------------|-------------------|------------------------|----------|----------------|
|                             |              |                   | Negative               | positive |                |
| Gender                      | Male         | 110               | 38                     | 72       | 1.000 (0.000)  |
|                             | female       | 6                 | 2                      | 4        |                |
| Age (y)                     | ≤ 60         | 48                | 19                     | 29       | 0.440 (0.597)  |
|                             | > 60         | 68                | 21                     | 47       |                |
| Tumor site                  | Supraglottic | 38                | 11                     | 27       | 0.598 (1.028)  |
|                             | glottic      | 68                | 26                     | 42       |                |
|                             | subglottic   | 10                | 3                      | 7        |                |
| Clinical stage              | I/II         | 44                | 21                     | 23       | 0.019 (5.504)* |
|                             | III/IV       | 72                | 19                     | 53       |                |
| T stage                     | T1/T2        | 53                | 20                     | 33       | 0.631 (0.230)  |
|                             | T3/T4        | 63                | 20                     | 43       |                |
| Tumor grade                 | G1           | 58                | 26                     | 32       | 0.046 (6.140)* |
|                             | G2           | 40                | 11                     | 29       |                |
|                             | G3           | 18                | 3                      | 15       |                |
| N stage                     | N0           | 51                | 24                     | 27       | 0.020 (5.417)* |
|                             | N+           | 65                | 16                     | 49       |                |



**Figure 2.** Fluorescent expression after transfection. ASONS: Antisense sequences; ASONS-C: mismatched sequences; Normal: cells without treatment as control. The white scale bars in figures are 50  $\mu$ m.

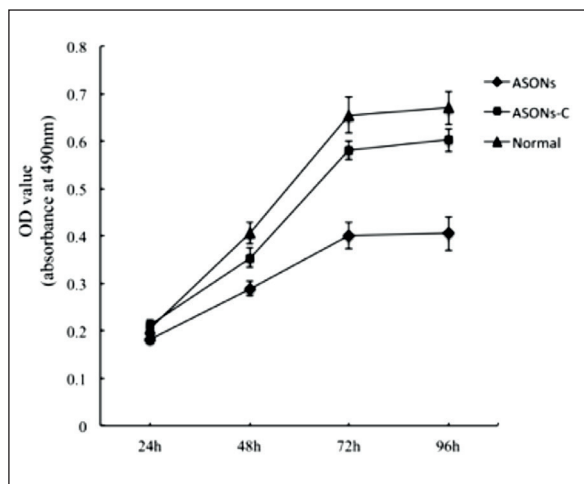
### Apoptosis of Hep-2 Cells after Treatment With ASONS

Flow cytometry analysis showed that Hep-2 cells transfected with ASONS for 48 h increased the percentage of apoptotic cells significantly compared with the control group ( $p < 0.05$ ). As shown in Figure 4, the early apoptosis rate of Hep-2 cells after transfection was  $42.22\% \pm 3.29\%$  for ASONS, which was significantly high-

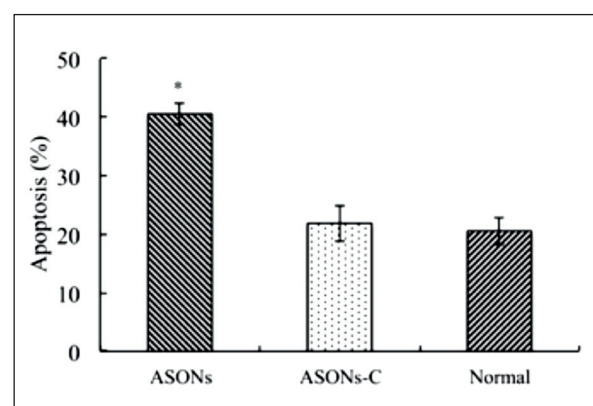
er than  $23.05\% \pm 3.52\%$  of mismatch sequence and  $20.10\% \pm 3.05\%$  of control cells.

### Changes in Epithelial and Mesenchymal Marker Gene Expression in ILK ASONS-Transfected Cells

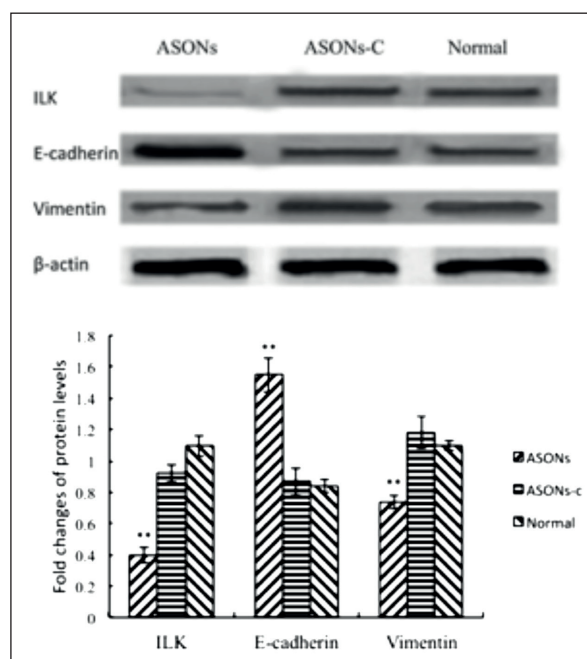
Changes in ILK, E-cadherin and Vimentin expression were examined by Western blot (Figure 5). The expression of ILK and the mesenchymal marker gene protein Vimentin were lower in ILK ASONS-transfected cells compared to ILK ASONS-C-transfected cells and normal control cells ( $p < 0.05$ ). The expres-



**Figure 3.** Effects of the knock-down of ILK on cell proliferation. The proliferation of Hep-2 cells was examined by MTT assay. ILK ASONS transfected cells showed significant decreases in cell proliferation from day 1 to day 4 after the ILK ASONS transfection. ASONS: Antisense sequences; ASONS-C: mismatched sequences; Normal: cells only treated with Lipofectamine 2000.



**Figure 4.** The apoptotic efficiency of Hep-2 cells transfected for 48 h with different oligonucleotides by flow cytometry. The apoptosis efficiency of ASONS-treated cells was significantly higher than control groups ( $p < 0.05$ ). For each experimental condition, the assay was performed in triplicate. ASONS: Antisense sequences; ASONS-C: mismatched sequences; Normal: cells only treated with Lipofectamine 2000.  $*p < 0.05$ .



**Figure 5.** Western blot for ILK protein expression levels in Hep-2 cells transfected with ILK ASONS compared to ASONS-C and Normal cells. The expression of E-cadherin was increased, coupled with reduced ILK and Vimentin expression in ILK ASONS transfected cells compared to the mock control. ASONS: Antisense sequences; ASONS-C: mismatched sequences; Normal: cells only treated with Lipofectamine 2000 as control.  $\beta$ -Actin was used as an internal loading control.  $**p < 0.05$  versus ASONS-C or Normal.

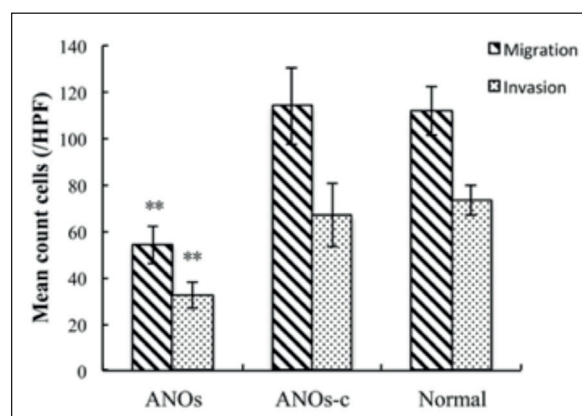
sion of the epithelial marker gene protein E-cadherin was higher in ILK ASONS-transfected cells compared to ILK ASONS-C-transfected cells and normal control cells ( $p < 0.05$ ).

#### The Inhibitory Effects of ASONS on Hep-2 Cell Migration Activity

In the transwell cell migration assay (Figure 6), the average cell count crossing PVPF filter in one HPF was  $54.3 \pm 8.2$  (mean  $\pm$  SD; triplicate determinations) for ILK ASONS-transfected cells, which was lower than  $113.9 \pm 16.7$  (mean  $\pm$  SD; triplicate determinations) for ASONS-C-transfected cells and  $111.7 \pm 10.4$  (mean  $\pm$  SD; triplicate determinations) for normal control cells ( $p < 0.01$ ). In conclusion, the cell migration activity of Hep-2 cells transfected with ILK ASONS was lower than that of ILK ASONS-C-transfected cells and normal control cells.

#### The Inhibitory Effects of ASONS on Hep-2 Cell Invasive Activity

In a Matrigel invasion assay (Figure 6), the average number of cells that invaded a Matri-



**Figure 6.** Effects of the knock-down of ILK on cell migration and invasive activity. The cells that appeared on the lower surface of the filter were counted in HPF. The data were obtained from three independent experiments. ASONS: Antisense sequences; ASONS-C: mismatched sequences; Normal: cells only treated with Lipofectamine 2000 as control.  $\beta$ -Actin was used as an internal loading control.  $**p < 0.05$  versus ASONS-C or Normal.

gel-coated membrane in one HPF was  $32.5 \pm 6.0$  (mean  $\pm$  SD; triplicate determinations) for ILK ASONS-transfected cells, which was lower than the  $66.9 \pm 13.5$  (mean  $\pm$  SD; triplicate determinations) cells for ILK ASONS-C-transfected cells and  $73.1 \pm 6.5$  (mean  $\pm$  SD; triplicate determinations) cells for normal control cells ( $p < 0.01$ ). The cell invasive activity of Hep-2 cells transfected with ILK ASONS was lower than that of ILK ASONS-C-transfected cells and normal control cells.

## Discussion

Over the past few decades, the treatment of laryngeal cancer with surgery, radiotherapy and chemotherapy have been greatly improved. However, no treatment has achieved satisfactory therapeutic outcomes and the survival rate of LSCC has not been significantly improved<sup>1</sup>. Therefore, it is important to understand the molecular mechanisms involved in the invasion and metastasis of LSCC and to identify new biomarkers.

ILK is an intracellular serine/threonine kinase linking integrin to the cell cytoskeleton by interacting with the cytoplasmic domains of integrin  $\beta 1$  and  $\beta 3$  subunits. The previous study showed increased ILK expression in many types of human tumors such as melanoma, colon, prostate, ovarian cancer, non-small cell lung cancer and head and neck squamous cell carcinomas<sup>4,13-15</sup>.



In our previous study<sup>11</sup>, for the first time, we demonstrated that increased ILK expression was significantly correlated with lymph node metastasis and poor survival rates in patients with LSCC. Our data have provided evidence that the ILK is required for tumor differentiation and metastasis in laryngeal carcinoma. In this work, we tested the inhibitory effects of ASONS targeting ILK on Hep-2 cells *in vitro*, aiming to investigate the possible mechanism of ILK in regulating proliferation, apoptosis, migration and invasion of laryngeal squamous cell carcinoma cells. As expected, we observed significant growth inhibition and apoptosis of Hep-2 cells after treatment with ASONS. In addition, ILK suppression by antisense ILK caused U87 glioma cell apoptosis and delayed tumor growth in Rag-2M mice bearing human U87 glioblastoma tumors<sup>16</sup>, suggesting that ILK is an important therapeutic target.

Because of the oncogenic properties of ILK, we also wanted to measure the inhibitory effect of ILK on the migration and invasion of Hep-2 cells. Tumor invasion and metastasis, whereby cancer cells escape from the primary tumor mass and colonize at remote locations, involve multiple steps, including localized invasion, intravasation, transport to other organs, extravasation, micrometastasis and colonization. Epithelial-mesenchymal transition (EMT), through which epithelial cells acquire characteristics of mesenchymal cells, is thought to play an important role in invasion and metastasis<sup>17</sup>.

E-cadherin and Vimentin are well-recognized proteins to be selectively expressed and play specific roles in epithelial and mesenchymal cellular states, respectively. E-cadherin, a member of the cadherin superfamily of calcium-dependent transmembrane glycoproteins, plays an essential role in normal physiological processes and pathological conditions such as EMT. The expression of E-cadherin, coupled with the reduced or absent expression of Vimentin, are epithelial-derived cell characteristics, whereas the reverse is a mesenchymal phenotype. Interestingly, this mesenchymal expression pattern is shown in cultured tumor cells that have a migratory and aggressive phenotype<sup>18-20</sup>.

The overexpression of ILK in a variety of cell lines such as 253J bladder cancer cells and IEC-18 cells has been shown to result in the downregulation of the epithelial markers E-cadherin, cytokeratin 18 and MUC1, and the upregulation of the mesenchymal markers LEF1, fibronectin and Vimentin<sup>21-23</sup>. In contrast, the suppression of the ILK activity in human tongue cancer cells down-

regulates expression of N-cadherin, Vimentin, Snail, Slug and Twist, and upregulates E-cadherin expression *in vivo* and *in vitro*<sup>24</sup>, which suggests that ILK can induce a complete EMT in various epithelial cell lines.

ILK inhibits the expression of E-cadherin through the activation of PKB phosphorylation and the induction of the transcription factor Snail and ZEB1 expression, and promotes tumor invasion and metastasis<sup>19,25</sup>. The downregulation of E-cadherin promotes a reduced cell-cell adhesion. Under confluent conditions, these cells detach, grow in suspension and undergo EMT, which is characterized by reduced E-cadherin expression and increased expression of Snail and Vimentin. The overexpression of ILK increases the invasive potential of cancer cells by stimulating the expression of invasion-related genes, such as MMP-9 and MMP2. The ILK overexpression increases the expression of MMP-9 *via* GSK-3 $\beta$  and activator protein transcription factors to increase intestinal and mammary epithelial cell invasiveness. ILK also stimulates the expression of MMP2 by PKB phosphorylation and promotes cell migration and invasion<sup>21,26</sup>. This study shows that silencing the ILK gene *in vitro* suppresses the proliferation, migration and invasion of LSCC cells from Hep-2 cell line by downregulating Vimentin expression and overexpressing E-cadherin, hindering the process of EMT. The decreased migration and invasion of Hep-2 cells may be associated with enhanced expression of E-cadherin.

## Conclusions

We detected that ILK plays a role in regulating laryngeal carcinomas differentiation and metastasis. The inhibition of the ILK expression reduces Hep-2 cells proliferation, migration and invasion. These findings suggest that the ILK gene could be a potential target for the treatment of laryngeal cancer.

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## Conflict of Interests

The authors declare that they have no conflict of interest.



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