LINC00941 promoted in vitro progression and glycolysis of laryngocarcinoma by upregulating PKM via activating the PI3K/AKT/mTOR signaling pathway

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Abstract

Background: LINC00941 has been proved to be related to various tumors, but its relationship with laryngocarcinoma remains vague.

Methods: LINC00941 expression in laryngocarcinoma tumor and laryngocarcinoma cells was determined by real time-quantitative polymerase chain reaction (RT-qPCR). Besides, the five-year survival of laryngocarcinoma patients with different LINC00941 expression was analyzed with Kaplan–Meier survival analysis, and the clinical characteristics of laryngocarcinoma patients were also recorded. After transfection, cell viability, cell proliferation, apoptosis, cell cycle, migration, and invasion were detected by cell counting kit-8 (CCK-8), colony formation, flow cytometry, cell scratch, and Transwell assays, respectively. Glycolysis was assessed by the colorimetric method. Expressions of proliferation-associated proteins, migration-associated proteins, glycolysis-associated proteins, and phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signal pathway-associated proteins were detected by Western blot.

Results: In laryngocarcinoma tumor tissues and cells, LINC00941 was highly expressed. High expression of LINC00941 decreased the 5-year survival of laryngocarcinoma patients, and it was positively related to lymph node metastasis and clinical stages. LINC00941 overexpression decreased apoptosis but promoted cell viability, proliferation, cell-cycle progression, migration, and invasion, and glucose consumption and lactate production in laryngocarcinoma cells. Moreover, LINC00941 overexpression elevated expressions of Ki-67, PCNA, MMP2, N-Cadherin, HK2, PFKFB4, and PKM, activated the PI3K/AKT/mTOR signal pathway but reduced E-Cadherin expression, while LINC00941 silencing had the opposite effects. PKM overexpression reversed the effects of LINC00941 silencing on cellular and glycolytic phenotypes.

Conclusion: LINC00941 promoted in vitro progression and glycolysis of laryngocarcinoma cells by upregulating PKM via activating the PI3K/AKT/mTOR signaling pathway.
1 | INTRODUCTION

According to the statistics, head and neck squamous cell carcinoma (HNSC) is the sixth most common cancer in the whole world, among which laryngocarcinoma is the most prevalent one. Laryngocarcinoma generally originates from the human skin of larynx and could lead to complications including cough, trachyphonia, dysphagia, and dyspnea, bringing about great pain to patients. Although the cure rate of patients with early-stage laryngocarcinoma tumor reaches 80% to 90%, the cure chance of advanced-stage patients is still as low as 60%. Since the early and effective diagnosis biomarkers of laryngocarcinoma are still unavailable, most patients with laryngocarcinoma are diagnosed at the advanced stage, and the mortality of patients with this disease remains high. In clinical practice, the five-year survival rate of advanced laryngocarcinoma patients was less than 50% due to the metastasis and poor prognosis. Therefore, for finding more effective treatment strategies, it is an urgent matter to understand in detail the underlying molecular basis.

The genome-wide studies revealed that noncoding RNAs (ncRNAs) make up 98% of the human genome. Although ncRNAs are incapable of encoding proteins, they are involved in conversed protein-coding feedback functions. Among them, long noncoding RNAs (lncRNAs) are ncRNAs with over 200 nucleotides. Over the past decade, increasing evidence has demonstrated that lncRNAs play critical roles in various developmental processes and diseases. Besides, previous studies have found that the expressions of many lncRNAs are abnormal in unclear pathogenesis and refractory tumors. LncRNAs generally controlled gene expression in multiple aspects such as transcription, translation, and protein functions, thus contributing to cell proliferation, apoptosis, invasion, metastasis, and other tumor-pertinent cellular processes. LINCO0941, also known as LncRNA-MUF, is a long intergenic non-protein-coding RNA, which has been proved to be a notable protumorigenic and prometastatic lncRNA in cancer biology. Growing studies verified that the expression of LINCO0941 was upregulated in various human malignant tumors including colorectal cancer, oral squamous cell carcinoma, pancreatic cancer, non-small-cell lung cancer, gastric cancer, and papillary thyroid cancer. Wu et al. proved the oncogenic effect of the upregulated LINCO0941 on colorectal cancer. Chen et al. proved that the abnormally high-expressed LINCO0941 in papillary thyroid cancer is linked to accelerated cancer malignant progression. Ai et al. also found the function of LINCO0941 in promoting the progression of oral squamous cell carcinoma. However, there is still lacking the evidence of the correlation between laryngocarcinoma and LINCO0941.

It is well recognized that tumors have an altered metabolic phenotype, the characteristics of which is that tumors tend to preferentially depend on glycolysis for energy production rather than on oxidative phosphorylation. Glycolysis promotes the glucose uptake and the conversion of glucose into pyruvate, followed by the production of lactate, and it also can facilitate the tumor growth. Xu’s study has discovered that LINCO0941 enhances aerobic glycolysis to promote pancreatic cancer cell growth, however, whether LINCO0941 enhances glycolysis in laryngocarcinoma remains unknown.

In this research, the LINCO0941 expression in laryngocarcinoma tumor tissues and cells was detected, the effects of LINCO0941 on laryngocarcinoma cell viability, proliferation, apoptosis, cell-cycle progression, the abilities of migration and invasion were also explored, and the effect of LINCO0941 on glycolysis in laryngocarcinoma cells was analyzed as well.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The Ethics Committee of the Taizhou Municipal Hospital (Z20200322-08Z) approved the procedures of the current study. In any experimental work associated with humans, written informed consents were obtained from all patients. All methods were adopted in accordance with the relevant guidelines.

2.2 | Clinical samples

Laryngocarcinoma tumor and adjacent tissue specimens were collected from 56 patients who received surgery at the Taizhou Municipal Hospital from 2013 to 2016. Clinical characteristics of patients including gender, age, differentiation, tumor depth, lymph node, and clinical stage were recorded, as listed in Table 1. All tumor tissue samples were frozen in liquid nitrogen immediately after surgery for later detection.

2.3 | Bioinformatics analyses

The expression pattern of LINCO0941 in HNSC was obtained from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/projects/TCGA-HNSC).

2.4 | Cell culture

Normal human laryngeal cell (HuLa-PC) line was obtained from American Type Culture Collection (CRL-3342, Manassas, VA, USA). AMC-HN-8 cell line (BFN60808789), one of the human laryngeal...
squamous cells, was acquired from BLUEFBIO (Shanghai, China); other human laryngeal squamous cell lines including SNU-46 (00046), SNU-899 (00899), and SNU-1076 (01076) cells were purchased from Korean Cell Line Bank (Seoul, Korean, http://cellbank.snu.ac.kr). All cells were cultured in the Dulbecco’s Modified Eagle Medium (DMEM; 10566016, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; C0256, Beyotime, Shanghai, China) and 1% Penicillin-Streptomycin-Glutamine (10378016, Thermo Fisher Scientific, Waltham, MA, USA) under a humid atmosphere with 5% CO₂ at 37°C.

### 2.5 | Transfection

The short hairpin RNA vector for LINC00941 (shLINC00941), the vector for LINC00941 overexpression, pyruvate kinase M (PKM) overexpression, and the corresponding empty vectors were synthesized by GenePharma (Shanghai, China). Before transfection, 2 × 10⁵ cells/ml SNU-46 and SNU-899 cells were seeded into a 6-well plate and cultured in DMEM with FBS. When the confluence of cells reached 80%, SNU-46 and SNU-899 cells in each well were incubated with 0.5-µg shLINC00941, LINC00941 overexpression vector, PKM overexpression vector, or a combination of shLINC00941 and PKM overexpression vector for transfection at 37°C for 48 h by Lipofectamine™ 2000 Transfection Reagent (11668027, Thermo Fisher Scientific, Waltham, MA, USA).

### 2.6 | Cell Counting Kit-8 (CCK-8) assay

We used CCK-8 (C0037, Beyotime, Shanghai, China) to measure the viability of SNU-46 and SNU-899 cells, respectively. According to the manufacturer’s instructions, after transfection with shLINC00941, LINC00941 overexpression vector, PKM overexpression vector, or a combination of shLINC00941 and PKM overexpression vector, the cells were digested by 0.25% trypsin (C0201, Beyotime, Shanghai, China), Following being washed and resuspended in DMEM with 10% FBS, 5 × 10³ cells/well SNU-46 and SNU-899 cells were inoculated in a 96-well plate to preculture for 24, 48, and 72 h. The cells were then incubated with 10-µl CCK-8 solution for 1 h at 37°C. Cell viability was measured by reading the optical density (OD) value via a microplate reader (Fluoroskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 450 nm.

### 2.7 | Colony formation assay

The shLINC00941 or LINC00941 overexpression-vector-transfected SNU-46 and SNU-899 cells in the log phase were digested and dispersed into a single-cell suspension by the clone formation medium consisting of DMEM and 10% FBS. After gradient dilution, culture dishes were added with a 10-ml clone formation medium and preheated to 37°C, and then, cell suspensions were seeded on culture dishes with the concentration of 500 cells/dish. Cells were then incubated with 5% CO₂ at 37°C for 2 weeks. After fixation with 4% paraformaldehyde (PFA; R37814, Thermo Fisher Scientific, Waltham, MA, USA), the visible colonies were dyed with 0.1% crystal violet (R40052, Thermo Fisher Scientific, Waltham, MA, USA), and the number was counted under a microscope (AxioLab 5, Zeiss, Oberkochen, Germany).

### 2.8 | Apoptosis assay

The apoptosis of SNU-46 and SNU-899 cells transfected with shLINC00941 and LINC00941 overexpression vector was determined by an Annexin V-FITC Apoptosis Detection Kit (C1062, Beyotime, Shanghai, China). Briefly, 5 × 10⁴ cells were added with 195-µl binding buffer and 5-µl Annexin V-FITC. After being incubated for 15 min in the dark, cells were washed with the binding buffer and then incubated with 10-µl propidium iodide (PI) for another 15 min in the dark. We used a flow cytometer (BD FACSCalibur, Becton-Dickinson, Franklin, NJ, USA) and FlowJo software (VX10, Tree Star, Ashland, OR, USA) to assess apoptosis.

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**Table 1** The relationship between LINC00941 expression and clinical characteristics

| Features               | No. of cases | LINC00941 expression | p value |
|------------------------|--------------|----------------------|---------|
| Age                    |              |                      |         |
| <60                    | 24           | 3.71 ± 2.37          | 0.188   |
| ≥60                    | 32           | 6.53 ± 10.12         |         |
| Gender                 |              |                      |         |
| Male                   | 45           | 5.11 ± 7.56          | 0.470   |
| Female                 | 11           | 3.42 ± 2.36          |         |
| Differentiation        |              |                      |         |
| Well                   | 16           | 3.16 ± 2.29          | 0.181   |
| Moderate               | 30           | 4.94 ± 8.41          |         |
| Poor                   | 10           | 8.55 ± 7.98          |         |
| Lymph node             |              |                      |         |
| Negative               | 36           | 2.83 ± 3.71          | 0.004** |
| Positive               | 20           | 8.76 ± 10.66         |         |
| Tumor depth (pT)       |              |                      |         |
| T1                     | 10           | 2.42 ± 2.07          | 0.187   |
| T2                     | 16           | 3.43 ± 5.97          |         |
| T3                     | 16           | 4.93 ± 5.67          |         |
| T4                     | 14           | 8.33 ± 11.32         |         |
| Clinical stage         |              |                      |         |
| I                      | 7            | 2.03 ± 1.66          | 0.006** |
| II                     | 13           | 1.90 ± 1.69          |         |
| III                    | 32           | 5.58 ± 6.62          |         |
| IV                     | 4            | 16.41 ± 20.36        |         |

**represents significant difference p < 0.01.
2.9 | Cell-cycle analysis

A cell-cycle analysis kit (C1052, Beyotime, Shanghai, China) and a flow cytometry (BD FACSCalibur, Becton-Dickinson, Franklin, NJ, USA) were used to measure the cell cycle of shLINC00941 or LINC00941 overexpression vector-transfected SNU-46 and SNU-899 cells. As the manufacturer recommended, cells were digested to disperse cells. The cells were incubated with PI staining buffer for 30 min at 37°C in the dark. After the cells were rinsed, the cell cycle was analyzed by flow cytometry at a wavelength of 488 nm and FlowJo software (VX10, Tree Star, Ashland, OR, USA).

2.10 | Cell scratch assay

The migration ability of SNU-46 and SNU-899 cells after transfection with shLINC00941, LINC00941 overexpression vector, PKM overexpression vector, or a combination of shLINC00941 and PKM overexpression vector was investigated by cell scratch assay. 2 × 10^4 transfected SNU-46 and SNU-899 cells were delivered in each well of 6-well plates, and the cells were cultured until 100% confluence was reached. A vertical horizontal scratch was made by a sterile pipette tip (TE-204-Y-L-R-S, Axygen, San Francisco, CA, USA). Later, the debris of cells was rinsed with PBS (C0221A, Beyotime, Shanghai, China), subsequent to which the cells were incubated with DMEM without serum. 0 and 48 h after incubation, an optical microscope (Axiolab 5, Zeiss, Oberkochen, Germany) was used to observe and capture the images of cells under the magnification of ×100.

2.11 | Transwell invasion assay

Transwell invasion assay was performed to measure the invasion ability of SNU-46 and SNU-899 cells after transfection with shLINC00941, LINC00941 overexpression vector, PKM overexpression vector, or a combination of shLINC00941 and PKM overexpression vector. The upper chamber of the 24-well Transwell chamber (Corning Incorporation, Corning, NY, USA) was precoated with 30-μl diluted Matrigel (356231, BD Biosciences, Franklin Lakes, NJ, USA), and the lower chamber was supplemented with 500-μl Roswell Park Memorial Institute (RPMI) 1640 (12633012, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS. Following being digested with 0.25% trypsin and washed with PBS, cells were resuspended in serum-free RPMI 1640. 200 μl of cell suspension (3 × 10^5 cells/ml) was then delivered to the upper chamber and cultured for 48 h at 37°C. The cells that failed to pass through the membrane were removed by cotton swabs, while the remaining cells passing through the membrane were fixed by 4% PFA (R37814, Thermo Fisher Scientific, Waltham, MA, USA) and stained with 0.1% crystal violet (R40052, Thermo Fisher Scientific, Waltham, MA, USA). A microscope (Axiolab 5, Zeiss, Oberkochen, Germany) was applied to count the number of invaded cells at ×250 magnification.

2.12 | Real time-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to measure the expressions of LINC00941, PKM, hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) and to assess the transfection efficiency of shLINC00941 and LINC00941. The laryngocarcinoma tumors/adjacent tissues or laryngocarcinoma cells were lysed by TRizol reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min to extract total RNA at first. The extracted total RNA was centrifuged at 12,000 g for 15 min at 4°C and was then incubated with 200-μl trichloromethane (T117625, Aladdin, Shanghai, China) for 5 min. After centrifugation for 5 min at 5000 g at 4°C, the supernatant liquid was added with 1 ml isopropl alcohol (9500–1, Thermo Fisher Scientific, Waltham, MA, USA) and then centrifuged again. The precipitated RNA was dissolved by DEPC-treated water (750024, Thermo Fisher Scientific, Waltham, MA, USA). In the reverse-transcribed process, cDNA was obtained by a PrimeScript RT reagent Kit (RR037A, Takara, Tokyo, Japan) according to the manufacturer's instructions. A TB Green Premix Ex Taq™ II (RR820A, Takara, Tokyo, Japan) were then employed in the RT-PCR. TB Green Premix Ex Taq II, ROX Reference Dye II, appropriate primer, cDNA, DEPC-treated water, and primers were then mixed uniformly. The sequences of forward (F) and reverse (R) primers on relevant genes were illustrated as follows: LINC00941 (F: 5′-GACCTTTTCAGCCACGGATT-3′, R: 5′-ACAATCTGGATAGAGGGCTCA-3′); HK2 (F: 5′-GAGCCACCCTCCACCTACT-3′, R: 5′-CCAGCCATGGCAATGTG-3′); PFKFB3 (F: 5′-TTGGCGTCCCCACAAAAGT-3′, R: 5′-AGTTTGGAGACGCTGTACCTGCTT-3′); PFKFB4 (F: 5′-GGAGTTCAATGTTGGCCAGT-3′, R: 5′-TCAGGATCCACACAGATGGA-3′); PKM (F: 5′-ATGTGCGGCTTCCCCACAAAGT-3′, R: 5′-AGTTTGGAGACGCTGTACCTGCTT-3′); GAPDH (F: 5′-CCACCTTCCAGGAGCGAGAT-3′, R: 5′-TGCTGATGATGGCTTATGCTA-3′). RT-qPCR was then conducted using a PCR instrument (Applied Biosystems 7500 Fast Dx Real-Time PCR instrument, Thermo Fisher Scientific, Waltham, MA, USA), and the conditions of amplification were listed below: pre-denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The corresponding gene expressions were quantified based on the 2^-ΔΔCt method.

2.13 | Western blot assay

The expressions of proliferation-associated proteins, migration-associated proteins, and PI3K/AKT/mTOR signal pathway-associated proteins in laryngocarcinoma cells with transfection with sh-LINC00941, LINC00941 overexpression vector, PKM overexpression vector, or a combination of shLINC00941 and PKM overexpression vector were measured by western blot. The proteins were extracted by lysis buffer (P0013J, Beyotime, Shanghai, China), and the protein concentration was measured by a BCA assay kit (P0012, Beyotime, Shanghai, China). 20-μl proteins were then loaded on 10% SDS polyacrylamide gel (P0012AC, Beyotime, Shanghai, China). After
The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (FP326, Beyotime, Shanghai, China). Next, the membranes were blocked with 5% nonfat milk at 37°C for 1 h, followed by incubation with the primary antibodies at 4°C overnight, including those against Ki-67 (ab92742, 1:5000, 359 kDa, Abcam, Cambridge, MA, USA), proliferative cell nuclear antigen (PCNA; ab18197, 1:1000, 29 kDa, Abcam), Matrix Metalloproteinase 2 (MMP2; ab37150, 1:1000, 72 kDa, Abcam), p-mTOR (ab109268, 1:1000, 289 kDa, Abcam), N-Cadherin (N-Cad; ab18203, 1:1000, 130 kDa, Abcam), AKT (#9272, 1:1000, 85 kDa, Cell signaling technology, Danvers, MA, USA), p-PI3K (#4228, 1:1000, 85 kDa, Cell signaling technology, Danvers, MA, USA), AKT (#9272, 1:1000, 60 kDa, Cell signaling technology, Danvers, MA, USA), p-AKT (#9271, 1:1000, 60 kDa, Cell signaling technology, Danvers, MA, USA), p-mTOR (ab2732, 1:2000, 289 kDa, Abcam), E-Cadherin (E-Cad; ab40772, 1:20,000, 29 kDa, Abcam), Metallopeptidase 2 (MMP2; ab37150, 1:1000, 72 kDa, Abcam), E-Cadherin (E-Cad; ab40772, 1:20,000, 29 kDa, Abcam), Metallopeptidase 2 (MMP2; ab37150, 1:1000, 72 kDa, Abcam), and Goat Anti-Mouse IgG (ab205719, 1:2000, 58 kDa, Abcam, Cambridge, MA, USA), phosphatidylinositol 3-kinase (PI3K; #4292, 1:1000, 85 kDa, Cell signaling technology, Danvers, MA, USA), p-PI3K (#4228, 1:1000, 85 kDa, Cell signaling technology, Danvers, MA, USA), AKT (#9272, 1:1000, 60 kDa, Cell signaling technology, Danvers, MA, USA), p-AKT (#9271, 1:1000, 60 kDa, Cell signaling technology, Danvers, MA, USA), mammalian target of rapamycin (mTOR; ab2732, 1:2000, 289 kDa, Abcam, Cambridge, MA, USA), p-mTOR (ab109268, 1:1000, 289 kDa, Abcam, Cambridge, MA, USA), AKT (#9272, 1:1000, 85 kDa, Cell signaling technology, Danvers, MA, USA), p-AKT (#9271, 1:1000, 85 kDa, Cell signaling technology, Danvers, MA, USA), AKT (#9272, 1:1000, 60 kDa, Cell signaling technology, Danvers, MA, USA), p-AKT (#9271, 1:1000, 60 kDa, Cell signaling technology, Danvers, MA, USA), p-mTOR (ab2732, 1:2000, 289 kDa, Abcam, Cambridge, MA, USA), and GAPDH (ab8245, 1:20,000, 36 kDa, Abcam, Cambridge, MA, USA). Afterwards, the membranes were washed by PBS to remove the redundant primary antibodies, and further incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies Goat Anti-Rabbit IgG (ab205718, 1:2000, Abcam), and Goat Anti-Mouse IgG (ab205719, 1:2000, Abcam) at room temperature for 2 h. An ECL kit (PO01B, Beyotime, Shanghai, China) was exploited for the detection of signal, and then, the signal was read and analyzed by a chemiluminescence imaging system (Chemidoc XRS+, Bio-Rad, Hercules, CA, USA) and Image J 1.5i (National Institutes of Health, Bethesda, MD, USA). All protein expression levels were normalized against GAPDH, and the value of the protein expression level in the Control group was defined as one.

2.14 | Glucose consumption and lactate production analyses

The culture media of SNU-46 and SNU-899 cells in 96-well plates were collected for the analyses of glucose consumption and lactate production in laryngocarcinoma cells. The analyses of these two glycolytic phenotypes were separately performed using a lactate assay kit (ab65330, Abcam, Cambridge, MA, USA) and a glucose assay kit (ab136955, Abcam, Cambridge, MA, USA) in accordance with the manufacturer’s instructions. A microplate reader (Luminoskan, Thermo Fisher Scientific, Waltham, MA, USA) was employed to detect the absorbance at 450 nm and 412 nm, respectively.

2.15 | Statistical analysis

All data of experiments were presented as mean ± standard deviation. The Kaplan–Meier method was used for survival analysis of laryngocarcinoma patients. The correlation between the expression of LINC00941 and clinicopathological features of the patients was analyzed with t test. The one-way analysis of variance (ANOVA) with Dunnet’s post hoc test was applied to compare the mean values between groups. GraphPad Prism 8 (San Diego, CA, USA) was used for all analysis. A p value < 0.05 indicated a statistically significant difference.

3 | RESULTS

3.1 | LINC00941 was high-expressed in laryngocarcinoma and HNSC, and high expression of LINC00941 was associated with the reduced five-year survival of laryngocarcinoma patients

The detection of LINC00941 expression in 56 patients’ laryngocarcinoma tumor and adjacent tissues demonstrated that (Figure 1A) LINC00941 expression level in laryngocarcinoma tumor tissues was notably higher than that in the normal laryngeal tissues (p < 0.001). We analyzed the data of HNSC, which were obtained from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/projects/TCGA-HNSC). The data of LINC00941 expression in HNSC were exhibited in Figure 1B. Consistently, LINC00941 was highly expressed in HNSC (p < 0.001). Besides, we dichotomized patients with HNSC into two groups, which were high LINC00941 expression level and low LINC00941 expression level groups, according to the median expression level. Through Kaplan–Meier survival analysis (Figure 1C), it was observed that the five-year survival of laryngocarcinoma patients with high LINC00941 expression level was lower than that of the patients with low LINC00941 expression level (p = 0.0299). The statistical analysis of the relationship between LINC00941 and clinicopathological characteristics in 56 patients with laryngocarcinoma was listed in Table 1. High expression of LINC00941 was positively related to lymph node metastasis (p = 0.006) and clinical stages (p = 0.011). In addition, the LINC00941 expression level in laryngocarcinoma cells was measured by RT-qPCR. As shown in Figure 1D, LINC00941 expression was upregulated in SNU-46, AMC-HN-8, SNU-899, and SNU-1076 cells as compared with that in the HuLa-PC cells (p < 0.001).

3.2 | LINC00941 enhanced cell viability and promoted the proliferation of laryngocarcinoma cells

To verify the transfection efficiency, the LINC00941 expression in SNU-46 and SNU-899 cells transfected with LINC00941 overexpression vector or shLINC00941 vector was detected by RT-qPCR. Figure 2A,B exhibited that compared with transfection of empty vector, shLINC00941 transfection downregulated LINC00941 expression in both SNU-46 and SNU-899 cells (p < 0.001), and conversely, LINC00941 vector transfection
**FIGURE 1** LINC00941 was high-expressed in laryngocarcinoma and HNSC, and high expression of LINC00941 reduced five-year survival rate of laryngocarcinoma. (A) LINC00941 expression in laryngocarcinoma tumor and adjacent tissues was detected by RT-qPCR. (B) Data analysis of LINC00941 expression in HNSC. The data were obtained from TCGA. (C) Kaplan–Meier survival analysis of laryngocarcinoma patients with high or low LINC00941 expression. (D) LINC00941 expression in normal human laryngeal cells (HuLa-PC) and laryngocarcinoma cells including SNU-46, AMC-HN-8, SNU-899, and SNU-1076 cells. GAPDH served as an inner reference in RT-qPCR. \( **p < 0.01; ^{++}p < 0.001; ^{vs.} Normal; ^{+} vs. HuLa-PC. \) (GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HNSC, head and neck squamous cell carcinoma; RT-qPCR, real time-quantitative polymerase chain reaction; shLINC00941, short hairpin RNA vector for LINC00941; TCGA, The Cancer Genome Atlas)

**FIGURE 2** LINC00941 enhanced viability of laryngocarcinoma cells and promoted colony formation of laryngocarcinoma cells. (A) LINC00941 expression in SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was detected using RT-qPCR. (B) LINC00941 expression in SNU-899 cells with shLINC00941 vector or LINC00941 vector transfection was measured by RT-qPCR. (C) Viability of SNU-46 cells with shLINC00941 vector or LINC00941 vector transfection was measured by CCK-8 assay. (D) Viability of SNU-899 cells with shLINC00941 vector or LINC00941 vector transfection was assessed by CCK-8 assay. (E,F) Colony numbers of SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector were evaluated by colony formation assays. (G,H) Colony numbers of SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector were evaluated by colony formation assays. GAPDH served as an inner reference in RT-qPCR. \( * p < 0.05; ^{*} p < 0.01, ^{**} p < 0.001; ^{vs.} Empty vector. \) (CCK-8, cell counting kit-8; RT-qPCR, real time-quantitative polymerase chain reaction; shLINC00941, short hairpin RNA vector for LINC00941)
upregulated LINC00941 expression \( (p < 0.001) \), indicating that in laryngocarcinoma cells, LINC00941 was successfully overexpressed and silenced. After that, the viability of transfected SNU-46 and SNU-899 cells was assessed (Figure 2C,D). When cells were cultured for 48 h and 72 h after transfection, the detection results uncovered that LINC00941 transfection increased the viability of SNU-46 and SNU-899 cells \( (p < 0.01) \), while shLINC00941 transfection decreased the viability of SNU-46 and SNU-899 cells \( (p < 0.05) \), when compared to the transfection with empty vector. Moreover, in colony formation assay, it could be observed that in comparison with transfection with the empty vector, LINC00941 transfection increased the colony number of SNU-46 and SNU-899 cells \( (p < 0.001) \), while shLINC00941 transfection resulted in the reduction in the colony number \( (p < 0.001) \, \text{Figure 2E-H} \). Overall speaking, LINC00941 overexpression enhanced cell viability and promoted proliferation of laryngocarcinoma cells, and LINC00941 silencing had the opposite effects on laryngocarcinoma cells.

### 3.3 | LINC00941 downregulated the expressions of proliferation-associated proteins

Here, the expression levels of Ki-67 and PCNA were detected to verify the effect of LINC00941 on laryngocarcinoma cell proliferation. As shown in supplementary Figure 1A-D, Ki-67 and PCNA expression levels in SNU-46 and SNU-899 cells transfected with shLINC00941 vector were lower than those in SNU-46 and SNU-899 cells transfected with empty vector \( (p < 0.001) \). Besides, the expression levels of Ki-67 and PCNA in LINC00941-transfected SNU-46 and SNU-899 cells were higher than those of the empty vector-transfected SNU-46 and SNU-899 cells \( (p < 0.001) \).

### 3.4 | LINC00941 inhibited the apoptosis of laryngocarcinoma cells while promoting the cell-cycle progression in laryngocarcinoma cells

The apoptosis rate of SNU-46 and SNU-899 cells was measured by apoptosis assay. As a result (Figure 3A-D), compared with empty vector transfection, the apoptosis rate in SNU-46 and SNU-899 cells \( (p < 0.001) \) was elevated by LINC00941 silencing, while being lessened by LINC00941 overexpression \( (p < 0.001) \). Flow cytometry analysis unraveled the cell-cycle progression of SNU-46 and SNU-899 cells after transfection (Figure 3E-H). The cells with shLINC00941 transfection were arrested in the G0/G1 phase, as the proportion of cells with shLINC00941 transfection in the G0/G1 phase was higher than that of the cells with empty vector transfection \( (p < 0.001) \). The proportion of LINC00941-transfected SNU-46 and SNU-899 cells in the G0/G1 phase was lower than that in the cells with empty vector transfection \( (p < 0.01) \). To sum up, LINC00941 silencing promoted apoptosis yet suppressed cell-cycle progression in laryngocarcinoma cells, and LINC00941 overexpression reduced apoptosis while accelerating cell-cycle progression in laryngocarcinoma cells.

### 3.5 | LINC00941 promoted cell migration and invasion of laryngocarcinoma cells

To assess the effects of LINC00941 on laryngocarcinoma cell migration and invasion, we did the cell scratch assay and a Transwell invasion assay. The figure and data of cell scratch assay (Figure 4A-D) indicated that compared with empty vector-transfected SNU-46 and SNU-899 cells, the migration rate of the shLINC00941-transfected cells was reduced \( (p < 0.001) \), while the migration rate of the LINC00941-transfected cells was augmented \( (p < 0.001) \). For Transwell invasion assay (Figure 4E-H), the invasion rate of SNU-46 and SNU-899 cells was decreased by shLINC00941 transfection \( (p < 0.001) \) but increased by LINC00941 transfection \( (p < 0.001) \), when compared to that of empty vector-transfected SNU-46 and SNU-899 cells. In a word, the migration and invasion abilities of laryngocarcinoma cells were suppressed by LINC00941 silencing, while being promoted by LINC00941 overexpression.

### 3.6 | LINC00941 increased MMP2 and N-Cad expressions, decreased E-Cad expression, and promoted glycolysis in laryngocarcinoma cells

The expression levels of migration-associated proteins were measured to further investigate the effect of LINC00941 on the migration of laryngocarcinoma cells. The data were exhibited in supplementary Figure 2A-D. When compared to transfection with the empty vector, shLINC00941 transfection downregulated the expression levels of MMP2 and N-Cad \( (p < 0.001) \) and upregulated the expression level of E-Cad \( (p < 0.001) \). On the contrary, LINC00941 transfection upregulated the expression levels of MMP2 and N-Cad in SNU-46 and SNU-899 cells \( (p < 0.001) \) but downregulated the expression level of E-Cad \( (p < 0.001) \).

The relationship between LINC00941 and glycolysis was also investigated in laryngocarcinoma. As shown in Figure 5A-L, we found that compared with empty vector transfection, shLINC00941 transfection inhibited glucose consumption and lactate production \( (p < 0.001) \), and downregulated the expressions of HK2, PFKFB4, and PKM \( (p < 0.05) \) in SNU-46 and SNU-899 cells, while LINC00941 overexpression promoted glucose consumption and lactate production \( (p < 0.001) \), and meanwhile upregulated the expressions of HK2, PFKFB4 and PKM \( (p < 0.05) \). There was no significant change in PFKFB3 expression in SNU-46 and SNU-899 cells before and after shLINC00941 transfection. But, PFKFB3 expression was increased by LINC00941 overexpression transfection in SNU-899 cells \( (p < 0.05) \). Thus, it could be concluded that LINC00941 silencing inhibited glycolysis, and LINC00941 overexpression promoted glycolysis in laryngocarcinoma.
**FIGURE 3** LINC00941 inhibited the apoptosis of laryngocarcinoma cells while promoting the cell-cycle progression in laryngocarcinoma cells. (A,B) The apoptosis of SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was analyzed by flow cytometry. (C,D) The apoptosis of SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector by was analyzed by flow cytometry. (E,F) Cell-cycle progression of SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was assessed by flow cytometry. (G,H) Cell-cycle progression of SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was assessed by flow cytometry. **p < 0.01; ***p < 0.001; * vs. Empty vector. (shLINC00941, short hairpin RNA vector for LINC00941)
FIGURE 4  LINC00941 promoted the migration and invasion of laryngocarcinoma cells. (A,B) The migration ability of SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was determined by cell scratch assay (magnification: x100; scale: 50 µm). (C,D) The migration ability of SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was detected by cell scratch assay (magnification: x100; scale: 50 µm). (E-F) The invasion ability of SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was assessed by Transwell invasion assay (magnification: x250; scale: 50 µm). (G-H) The invasion ability of SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector was determined by Transwell invasion assay (magnification: x250; scale: 50 µm). ***p < 0.001; * vs. Empty vector. (shLINC00941, short hairpin RNA vector for LINC00941)
LINC00941 enhanced the viability and promoted the migration, invasion, and glycolysis of laryngocarcinoma cells by upregulating the PKM level

To determine how PKM expression is related to the effect of LINC00941 on laryngocarcinoma progression, we either transfected or co-transfected SNU-46 and SNU-899 cells with sh-LINC00941 and PKM vector. From the results revealed in Figure 6A,B, we discovered that the expression of PKM was lower in SNU-46 and SNU-899 cells transfected with shLINC00941 than that in SNU-46 and SNU-899 cells transfected with empty vector \((p < 0.001)\), and the expression of PKM in SNU-46 and SNU-899 cells co-transfected with PKM and shLINC00941 was higher than that in SNU-46 and SNU-899 cells transfected with shLINC00941 alone \((p < 0.001)\) but was lower than that in SNU-46 and SNU-899 cells transfected with PKM alone \((p < 0.001)\). After being

**Figure 5** LINC00941 promoted glycolysis in laryngocarcinoma cells. (A–D) The glucose consumption and lactate production of SNU-46 and SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector were assessed by the colorimetric method. (E–L) The expressions of HK2, PFKFB3, PFKFB4 and PKM in SNU-46 and SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector were measured by RT-qPCR assay. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\); * vs. Empty vector (HK2, hexokinase 2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; PKM, pyruvate kinase M; RT-qPCR, real time-quantitative polymerase chain reaction; shLINC00941, short hairpin RNA vector for LINC00941)
incubated for 48 and 72 h, SNU-46 and SNU-899 cells with PKM and shLINC00941 co-transfection exhibited increased OD values, as compared with SNU-46 and SNU-899 cells with shLINC00941 transfection (p < 0.01), and the OD values of SNU-46 and SNU-899 cells with PKM and shLINC00941 co-transfection were lower than those of SNU-46 and SNU-899 cells transfected with PKM (p < 0.001) (Figure 6C-D). Moreover, as exhibited by the results from scratch assay and Transwell invasion assay (Figure 6E-F and Figure 7A-C), the migration and invasion rates of PKM and shLINC00941-co-transfected cells were elevated (p < 0.001), as compared with these rates of shLINC00941-transfected SNU-46 and SNU-899 cells, and these rates of PKM and shLINC00941-co-transfected SNU-46 and SNU-899 cells were lower than the migration and invasion rates of PKM-transfected SNU-46 and SNU-899 cells (p < 0.001).

In addition, the data in Figure 7D-G uncovered that in comparison with transfection of shLINC00941 alone, co-transfection of PKM and shLINC00941 promoted glucose consumption and lactate production in SNU-46 and SNU-899 cells (p < 0.001). Additionally, the levels of glucose consumption and lactate production in SNU-46 and SNU-899 cells co-transfected with shLINC00941 and PKM were lower than those in SNU-46 and SNU-899 cells transfected with PKM vector (p < 0.001). Taken together, these results suggested that LINC00941 could upregulate the PKM level to promote glycolysis and laryngocarcinoma progression.

3.8 | LINC00941 activated the PI3K/AKT/mTOR signaling pathway in laryngocarcinoma cells

Whether LINC00941 activated the PI3K/AKT/mTOR signaling pathway was explored in laryngocarcinoma. Through western blot assay (Figure 8A,B,F,G), we found that compared with empty vector transfection, shLINC00941 transfection downregulated p-PI3K, p-AKT and p-mTOR expressions in SNU-46 and SNU-899 cells (p < 0.001), while LINC00941 transfection upregulated p-PI3K, p-AKT and p-mTOR expressions (p < 0.001). Meanwhile, no significant change in expressions of PI3K, AKT and mTOR was detected in SNU-46 and SNU-899 cells before and after transfection with shLINC00941 or LINC00941. Besides, p-PI3K/Pi3K, p-AKT/AKT, and p-mTOR/mTOR in SNU-46 and SNU-899 cells were all decreased by shLINC00941 transfection, compared to those in SNU-46 and SNU-899 cells with empty vector transfection (p < 0.01, Figure 8C-E,H-J), while p-PI3K/Pi3K, p-AKT/AKT and p-mTOR/mTOR were all increased in SNU-46 and SNU-899 cells after LINC00941 transfection relative to empty vector transfection (p < 0.001, Figure 8C-E,H-J). Thus, it could be concluded that the PI3K/AKT/mTOR signaling pathway was inhibited by LINC00941 silencing while being activated by LINC00941 overexpression.

4 | DISCUSSION

Laryngocarcinoma accounts for about 25 to 30% of head and neck cancer-related cases worldwide. Laryngocarcinoma is a typical HNSC that is usually diagnosed at an advanced stage and has an unsatisfactory prognosis accompanied by cervical lymph node metastases and distant metastases. To design new therapeutic strategies and improve clinical outcomes, it is necessary to further investigate the molecular mechanisms of laryngocarcinoma. In addition, several studies have reported that lncRNAs such as TUG1, NEAT1, and PCAT9 contributed to the development of laryngocarcinoma. LncRNAs are non-protein-coding RNA transcripts longer than 200 nucleotides, which act as pivotal regulators of gene expression at the level of post-transcriptional and chromatin modification. It has been demonstrated that IncRNAs play vital roles in the regulation of assorted diseases. Growing evidence is emerging to show that a high expression level of IncRNA LINC00941 is related to various tumor progression, but the effect of LINC00941 on laryngocarcinoma is rarely studied. We detected the LINC00941 expression level in laryngocarcinoma tumor tissues and laryngocarcinoma cells, and the data displayed that LINC00941 was highly expressed in laryngocarcinoma tumor tissues and laryngocarcinoma cells. Interestingly, HNSC data from TCGA exhibited the similar results. Besides, we also found that the patients with high LINC00941 expression had poor prognosis and LINC00941 expression has a positive correlation with lymph node metastasis and clinical stages. The data revealed that LINC00941 expression was related to laryngocarcinoma, and high expression of LINC00941 yielded a promotive effect on laryngocarcinoma progression.

To further elucidate the potential effect and underlying mechanism of LINC00941 on laryngocarcinoma cells, we determined the viability of laryngocarcinoma cells with LINC00941 overexpression or LINC00941 silencing. The results of our study indicated that LINC00941 overexpression increased cell viability and proliferation yet reduced apoptosis of laryngocarcinoma cells, while
LINC00941 silencing had the opposite effects, which were in line with the findings regarding the tumor growth-promoting role of LINC00941 in oral squamous cell carcinoma, non-small-cell lung cancer, gastric cancer, and pancreatic cancer. Moreover, we measured proliferation-associated protein expressions in laryngocarcinoma cells to further prove the ability of LINC00941 to promote the proliferation of laryngocarcinoma cells. Ki-67 protein is a nuclear DNA-binding protein that is expressed in all the vertebrates. Numerous reports have demonstrated that Ki-67 only exists in all active phases (containing G1, S, and G2) of the cell-cycle progression, which is widely used as a tumor proliferation marker. Another proliferation-associated protein we measured in this study was PCNA, which is involved in cell proliferation, apoptosis, and repair. PCNA, as a pleiotropic protein, is an essential co-factor for DNA polymerases during replication, playing a critical role in DNA replication and replication-associated processes. After determination, the results showed that LINC00941 positively regulated Ki-67 and PCNA expressions, which was verified again that LINC00941 promoted laryngocarcinoma cell proliferation.

As mentioned above, laryngocarcinoma is often associated with metastasis, of which the initial step is cell migration and invasion. The results of cell scratch assay and Transwell invasion assay validated that the migration and invasion of laryngocarcinoma cells were promoted by LINC00941 overexpression, which, however, were suppressed by LINC00941 silencing. We also measured the expressions of migration-associated proteins, including MMP2, E-Cad, and N-Cad. MMPs are able to degrade various components of extracellular matrix proteins including the basement membrane and the surrounding stroma, among which MMP2 is a gelatinase that exerts an essential effect on growth initiation and tumor invasion in squamous cell carcinoma. Cadherin also plays a pivotal role in cell migration as a regulator in cell migration and invasion. Previous studies have demonstrated that E-cad is an invasion suppressor, and its level is downregulated in squamous cell carcinoma. While N-cad is an invasion promoter whose level is frequently upregulated. LINC00941 overexpression promoted migration and invasion, and upregulated MMP2 and N-cad expressions yet downregulated E-cad expression in laryngocarcinoma cells, while LINC00941 knockdown had the opposite effects. On the whole, the migration and invasion abilities of laryngocarcinoma cells were promoted by LINC00941, which was consistent with how LINC00941 promoted the in vitro progression of colorectal cancer, pancreatic cancer, and gastric cancer.

Cancer cells exhibit a glycolytic phenotype, which contributes to cancer cell survival and metastasis, and drug resistance related to impaired oxidative phosphorylation. LINC00941, which is highly expressed in pancreatic cancer, has been revealed to enhance glycolysis by promoting glucose consumption and lactate production, thus exacerbating pancreatic cancer cell proliferation. Consistent with the enhancing role of LINC00941 on glycolysis in pancreatic cancer, LINC00941 was found in our study to positively regulate glucose consumption and lactate production, which are indicative of glycolysis, in laryngocarcinoma cells.

To further ascertain the enhancing role of LINC00941 on glycolysis in laryngocarcinoma cells, we measured the expressions of rate-limiting enzymes key to glycolysis in laryngocarcinoma cells. HK2 is a cytoplasmic enzyme that can catalyze the first rate-limiting step of glucose metabolism and facilitate the utilization of adenosine triphosphate (ATP). PFKFB3 and PFKFB4 are two of the isozymes and allosteric activators of phosphofructo kinase (PFK), a controller of the glycolytic flux. PFKFB3 mainly catalyzes the synthesis of fructose-2,6-bisphosphate to promote the glycolytic flux. PFKFB4 redirects more glucose into the pentose phosphate pathway, further inducing ROS detoxification and the synthesis of lipid and nucleotide to promote cell proliferation. PKM is also a rate-limiting enzyme that catalyzes the final step of the glycolytic flux to generate pyruvate and ATP. Our results demonstrated that LINC00941 positively regulated the expressions of HK2, PFKFB4, PKM, and PFKFB3, though probably with no significant effect on PFKFB3 expression. These findings still affirmed that LINC00941 could enhance glycolysis in laryngocarcinoma cells. Notably, our study detected that the increase in PKM expression in laryngocarcinoma cells after LINC00941 overexpression was the most evident among the increase in the expressions of the other tested rate-limiting enzymes, hinting that PKM may play a predominant role in laryngocarcinoma cell glycolysis enhancement induced by LINC00941. The correctness of this hint was then confirmed by our results that overexpression of PKM reversed the LINC00941 knockdown-induced inhibition on glycolytic phenotypes in laryngocarcinoma cells. Moreover, our study depicted that PKM overexpression also reversed LINC00941 knockdown-induced suppression on laryngocarcinoma cell viability, migration, and invasion, and in return LINC00941 knockdown revered the effect of PKM overexpression on these cell phenotypes, signifying that the effect of LINC00941 on promoting laryngocarcinoma progression may be attributed to PKM overexpression.

PI3K, AKT, and mTOR have close inter-connections and PI3K-Akt-mTOR pathway is always considered a unique master regulator for diverse cancers. It has been demonstrated that the PI3K/AKT/mTOR signal pathway regulates cell survival, proliferation, and growth
FIGURE 8 LINC00941 activated the PI3K/AKT/mTOR signaling pathway in laryngocarcinoma cells. (A–E) The expressions of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in SNU-46 cells transfected with shLINC00941 vector or LINC00941 overexpression vector were measured by western blot assay. (F–J) The expressions of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in SNU-899 cells transfected with shLINC00941 vector or LINC00941 overexpression vector were detected by western blot assay. GAPDH served as an inner reference in western blot assay, **p < 0.01; ***p < 0.001. vs. Empty vector. (GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; shLINC00941, short hairpin RNA vector for LINC00941)

of advanced or recurrent endometrial cancer.\textsuperscript{57} It is often associated with tumor progression, which is activated in the majority of human malignant tumors.\textsuperscript{58} Besides, it plays a vital role in regulating glycolysis.\textsuperscript{59} Intracellular mTOR, which is activated via PI3K activation, could further phosphorylate Akt via the S473 sites, resulting in complete Akt activation. To the best of our knowledge, Akt activation controls the rates of glucose uptake into the cell via the glucose transporter 1(GLUT1).\textsuperscript{59} Also, the PI3K/AKT pathway can strengthen glycolysis by regulating fructose 2,6-bisphosphatase (PFKFB2) expression. Herein, we confirmed the effect of LINC00941 on activating the PI3K/AKT/mTOR signaling pathway in laryngocarcinoma cells, as suggested by the result indicated that LINC00941 could positively regulate the phosphorylation of PI3K, AKT, and mTOR in laryngocarcinoma cells. Taken together, LINC00941 is highly expressed in laryngocarcinoma. LINC00941 promotes cell viability, proliferation, cell-cycle progression, cell migration, and invasion and glycolysis but inhibits the apoptosis of laryngocarcinoma cells. Besides, PKM expression is essential to LINC00941-induced glycolysis of laryngocarcinoma cells. Furthermore, LINC00941 upregulates PKM expression and activates the PI3K/AKT/mTOR signaling pathway, via which LINC00941 promotes the in vitro progression and glycolysis of laryngocarcinoma cells.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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