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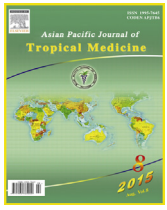

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Correlation of survivin, p53 and Ki-67 in laryngeal cancer Hep-2 cell proliferation and invasion

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ABSTRACT

Objective: To investigate the mechanism of survivin, p53 and Ki-67 on Hep-2 human laryngeal cancer endothelial cell proliferation and invasion.
Methods: Laryngeal squamous cell carcinoma and paracancerous normal tissues were collected, total RNA was extracted from tissues, *survivin*, *p53* and *Ki-67* gene mRNA expression levels in laryngeal cancer and the adjacent tissues were detected by Real-time PCR. Human laryngeal cancer Hep-2 epithelial cells were selected, *survivin* gene was overexpressed, and cell proliferation was detected by MTT. *p53* and *Ki-67* gene expression changes in overexpressed *survivin* gene were detected by Western blot. Changes in Hep-2 cell invasive ability were studied when *survivin* was overexpressed as detected by Transwell invasion assay.
Results: In the adjacent tissues, *survivin*, *p53* and *Ki-67* gene relative expression levels were 1.72 ± 0.9, 13.7 ± 5.7 and 5.7 ± 1.3, respectively; while in cancer tissues, gene relative expression levels were 53.7 ± 8.3, 66.7 ± 5.2 and 61.0 ± 3.1, respectively, which was significantly increased. As detected by MTT, relative cell survival rate within 12 h of *survivin* overexpression were: load control group (88.5 ± 1.6)%; overexpressed group (90.3 ± 1.9)%. Transwell invasion assay results indicated that overexpressed *survivin* could significantly increase the relative survival rate of cells.
Conclusions: Expressions of *p53*, *Ki67* and *survivin* are increased in cancer; and there is a positive correlation between *survivin*, *p53* and *Ki67* expressions in laryngeal carcinoma.

1. Introduction

Laryngeal cancer is a common malignancy that is secondly high estin disease incidence only after nasopharyngeal cancer in otolaryngology. Although the occurrence of cancer is a multifactorially complex process, it is generally believed that in cancer gene activation, tumor suppressor gene inactivation and overexpression of anti-apoptotic genes are important causes of cancer [1–3].

In recent years, *survivin* has been found to be an apoptosis inhibiting gene. It is a member of the apoptosis inhibitory protein factor family, which is tumor specific and only expressed in tumor and embryonic tissues; because it is expressed in a variety of tumors and highly correlated with tumor diffusion transfers. Therefore, it has become a hot topic in cancer research. Studies have found that survivin upregulation and p53 overexpression are closely related to nasal type NK/T-cell lymphoma. Survivin might prompt p53 to coordinate cell cycle regulation. Ki67 is a kind proliferation associated with nuclear antigens. In addition, it is expressed to all G_0 phase cell cycles, and closely related with tumor differentiation, invasion and metastasis [4–8]. Therefore, in this study, we discuss survivin with p53 and Ki-67 expressions in laryngeal carcinoma and its correlation with Hep-2 cell proliferation and invasiveness in tissue and cell levels; in order to explore the role of survivin in tumor occurrence and development.

2. Materials and methods

2.1. Materials

2.1.1. Hep-2 cell line

Hep-2 cell line was purchased from ATCC, and stored in liquid nitrogen out of laboratory. Cells were cultured in DMEM

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complete medium supplemented with 10% fetal bovine serum (Lanzhou MinHai Bio-engineering Co., Ltd) at 37 °C with a 5% CO_2 environment.

2.1.2. Drug reagents and equipment

Tissue RNA extraction kit (RNeasy Plus Mini Kit) was purchased from QIAGEN; Reverse transcription kit (iScriptcDNA Synthesis Kit) was purchased from Bio-Rad; Real-time fluorescence quantitative PCR kit (SsoAdvanced SYBR Green Super mix) was purchased from Bio-Rad; Transfection Reagent lipofectamine 2000 was purchased from Invitrogen; MTT kit was purchased from Promega; Survivin monoclonal antibody was purchased from Abcam; p53 and Ki67 antibodies were purchased from Santa Cruz Biotechnology; Transwell chamber was purchased from Millipore; Matrigel matrix (5 mg/mL) was purchased from BD.

CO_2 incubator was from SANYO; UV spectrophotometer (SmartSpec 3000) was from Bio-Rad; Shimadzu (Precision Balances) was from Sartorius; Fluorescence quantitative PCR detection system (CFX96 Touch) was from Bio-Rad.

2.2. Methods

2.2.1. Clinical tissue samples

Surgical specimens from 64 cases of laryngeal squamous cell carcinoma and 34 cases of the adjacent normal tissues were collected from January 1997 to January 2009 at the Department of Pathology, Affiliated Hospital of Hebei Engineering University. Among the 64 cases of laryngeal cancer patients, 52 were male and 12 were female; meanwhile, 45 of them were ≤60-years-old and 19 were >60-years-old, with the average age of these patients being 57.6. Clinical staging was based on the Union for International Cancer Control 1997 standards; accordingly, 9 cases were stage-I, 20 cases were stage-II, 28 cases were stage-III, and 7 cases were stage-IV. Among these cases, 31 cases had cervical lymph node metastasis, while 33 cases were without cervical lymph node metastasis. After the follow-up for 3–10 years, there were 14 deaths and 50 survival patients in 3 years, and two lost cases and 46 survival patients in 5 years.

Mortar was added liquid nitrogen and tissues were grinded to powder. Tissue RNA extraction kit was used to obtain total RNA and preserved in a freezer at −80 °C.

2.2.2. Real-time PCR assay method

Clinical cancer and the adjacent normal tissue samples were collected, and separately randomized three cases into a single sample. *Survivin*, *p53* and *Ki67* gene mRNA expression levels were detected in tissue total RNA of all samples. According to reverse transcription kit instructions, tissue total RNA was reverse transcribed to cDNA, and related genes were detected by real-time PCR. *Survivin*, *p53* and *Ki67* gene mRNA sequences were queried from the NCBI database to design Real-time PCR primers. All primers were synthesized by SBS Genetech Co., Ltd. Specific sequences are as follows (Tables 1–3). Gene amplification Ct value was determined by Real-time PCR, and Ct values were negatively correlated to the initial DNA copy number. *GAPDH* was treated as internal control using the relative quantification method. For the homogenization process, each sample was analyzed by the ΔCt value; ΔCt = target gene Ct-reference gene Ct.

2.2.3. Plasmids construction and cell transfection

Survivin expression plasmid coding sequences obtained from the *survivin* gene were inserted between the two restriction sites, *Bam*H I and *Xho* I, in the pcDNA3.1 plasmid (added EGFP tag to detect the expression). Plasmid constructs were carried out by the Beijing ComWin Biotech Co., Ltd.

An appropriate number of cells were inoculated through culture plates, and 500 μL of complete medium was added into each well, causing transfection cell density to reach 70%–80%. Using 50 μL of serum-free medium, 1 μL of Lipofectamine 2000 was diluted. Then, the diluted plasmid and Lipofectamine 2000 were mixed, and incubated at room temperature for 20 min. After 4–6 h of culture, replace with fresh complete medium, and continued to culture cells.

2.2.4. Cell proliferation detected by MTT

In accordance with the manufacturer's instructions of the MTT Kit (Promega, G3582), a small amount of CellTiter 96® Aqueous One Solution Reagent was directly added into culture wells and incubated for 1–4 h, and absorbance was recorded on a 96-plate reader at 490 nm. At 490 nm, the measured absorbance values and the number of living cells in cultures was directly proportional.

2.2.5. Western-blotting

Cells were collected and lysed using 50 μL of RIPA lysis buffer, simultaneously added with a protease inhibitor cocktail (added 10 μL of cocktail to 1 mL RIPA by volume ratio), and mixed by pipetting. After placed on ice for 30 min, cells were sonicated, and ultrasound probes were used to deliver a short shock on ice at an appropriate frequency, cleavage cocktail at 4 °C, and centrifuged at 13000 r/min for 20 min. The

Table 1
Primers used in real-time PCR.

Gene	Accession no.	Primer (5′–3′)
BIRC5	NM_001012271.1	For: TGTCATAGAGCTGCAGGGTG Rev: GTCGAGGAAGCTTTCAGGTG
TP53	NM_001003210.1	For: TCAACAAGATGTTTTGCCAACTG Rev: ATGTGCTGTGACTGCTTGTAGATG
MKI67	NM_001145966.1	For: CCACACTGTGTCGTCGTTTG Rev: CCGTGCGCTTATCCATTCA
GAPDH	NM_002046	For: TGGAAGGACTCATGACCACA Rev: TTCAGCTCAGGGATGACCTT

BIRC5, baculoviral IAP repeat-containing 5; TP53, tumor protein p53; MKI67, antigen identified by monoclonal antibody Ki-67; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; For, forward; Rev, reverse.

Table 2
Synthetic system of inverse transcription.

Components	Volume per reaction
5× iScript reaction mix	4 μL
iScript reverse transciptase	1 μL
RNA template (1 μg RNA)	1 μg
Nuclease-free water	Up to 20 μL

iScript reaction mix was purchased from Bio-Rad, Nuclease-free water was purchased from Invitrogen.

supernatant was placed into a new centrifuge tube, and protein concentration was determined using a protein assay kit.

The gel was immersed in transfer buffer for 10 min in equilibrium, the transfer 'Sandwich' assembled, transfer buffer was added, and the electrode was plugged at 100 V for 45–60 min. After the end of the membrane transfer, the PVDF membrane was rinsed with TBS for 10–15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk, shaken at room temperature for one hour, added with appropriate primary antibody dilution [TBST dilution containing 1% (w/v) skim milk], incubated for two hours at room temperature, and membrane was rinsed three times with TBST every 5–10 min. Then, the membrane was incubated in TBST diluted secondary antibody (1:10000, HRP labeled) containing 0.05% (w/v) nonfat dry milk at room temperature for one hour, and rinsed three times with TBST every 5–10 min. Exposed photographs of the experimental results were saved. The experiment was repeated three times. Quantity one v4.62 software was used to determine the molecular strip gray scale value (strip track quantitative method, Trace Tracking) according to different electrophoretic bands; then, optical density values were plotted on the optical density curve. Then, optical density was calculated according to the area under the curve as quantitative electrophoretic bands, and statistical analysis.

2.2.6. Bioinformatics analysis

Bioinformatics is an important method in studying protein interactions and the protein signaling pathway. Line analysis tool STRING 9.05 and the protein signaling pathway database, and KEGG PATHWAY Database (http://www.genome.jp), were used for protein interactions. As preliminarily analyzed, survivin, p53 and Ki67 molecules are involved in the signaling pathway and interacting proteins. The STRING database (http://string-db.org) integrated experimental research data and PubMed abstracts containing relevant results and bioinformatics method forecast results, which are search systems for known or predicted protein interactions.

Table 3
Synthetic system of PCR.

Components	Volume per reaction
SsoAdvanced SYBR Green Super mix	5 μL
Forward primer (10 μM)	0.3 μL (300 nM)
Reverse primer (10 μM)	0.3 μL (300 nM)
cDNA template	100 ng
Nuclease-free water	Up to 10 μL

SsoAdvanced SYBR Green Super mix was purchased from Bio-Rad.

2.2.7. Cell invasion relevant experiments

Matrigel stored at −20 °C was taken out for melting at 4 °C overnight. Matrigel was diluted with pre-cooled serum-free DMEM medium to a final concentration of 1 mg/mL, 100 μL of diluted Matrigel was added on the bottom of the central chamber of the Transwell, and incubated at 37 °C through gel. For gum reconstruction, 200 μL of DMEM culture medium was added into each well. After cells were trypsinized, centrifuged to remove the culture medium, washed cells with PBS, resuspended cells with serum-free medium, and cells were seeded on the upper chamber of the Transwell. Culture medium containing 10% FBS was added into the lower chamber and cultured cells at 37 °C. After the culture was completed, liquid in the upper chamber was discarded, the upper chamber was removed, and a cotton swab was used to wipe off those that did not pass through the cell membrane. Fixed in 3.7% formaldehyde for 10 min, crystal violet stained, and cells were observed under an inverted microscope. Three horizons were randomly selected, and the number of cells that passed through pores was calculated for statistical analysis.

2.3. Statistical analysis

SPSS12.0 statistical software was used for analysis. Counted data were analyzed by *t*-test, χ^2 test and correlation test using Spearman correlation analysis. $P < 0.05$ means that the difference was statistically significant.

3. Results

3.1. Survivin, p53 and Ki67 gene mRNA expression levels in clinical tissue samples detected by real-time PCR

As shown in Figure 1, in adjacent tissues, *survivin*, *p53* and *Ki67* genes relative expression levels were 1.72 ± 0.9; 13.7 ± 5.7; 5.7 ± 1.3; while in cancer tissues, expression levels of the three genes were significantly upregulated, and the relative expression levels were: 53.7 ± 8.3; 66.7 ± 5.2; 61.0 ± 3.1 ($P < 0.01$).

3.2. Survivin overexpression and MTT detection of cell apoptosis

Eukaryotic expression plasmids of *survivin* genes were transfected by Hep-2 cells, EGFP expressions were detected after 12 h, and significant green fluorescent protein expressions can be observed in cells. As explained, *survivin* genes have been normally expressed. Hep-2 cells that expressed for 24 h were collected, *survivin* was detected by Western blot, and it could be observed that the over-expression group was capable of expressing by exogenous *survivin*; while for the no-load group, endogenous expressions were only detected. Further, to detect *survivin* gene on cell proliferation, relative survival rate of the cells in *survivin* overexpression within 12–48 h was detected by MTT. That was, absorbance (490 nm) of first seed cells was used as a standard to detect cell viability at relatively different time periods. As explained, at a 12–48 h period, *survivin* gene transfected cells; and relative survival rate was higher in the control group, prompting *survivin* gene by regulating relevant pathways, and suppressing normal apoptosis (Figure 2).

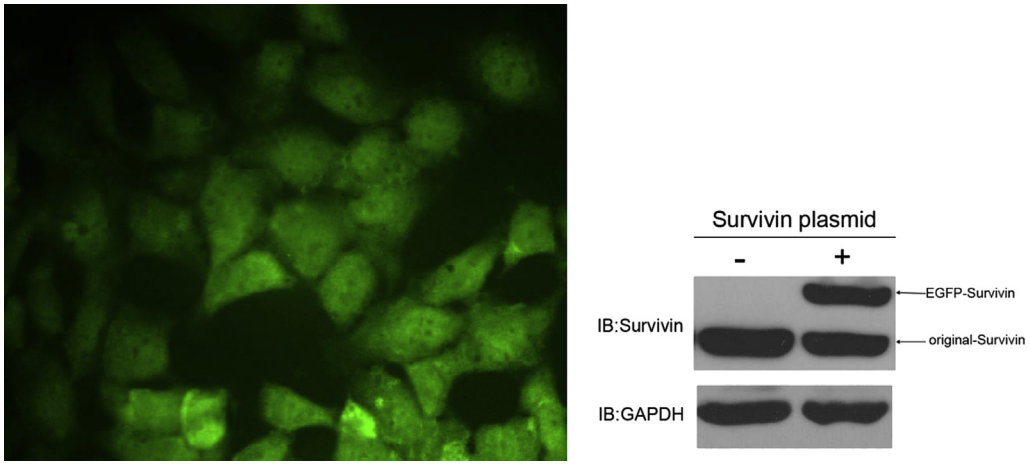

Figure 1. *Survivin* gene overexpression after 12 h of transfection is shown. Fluorescence microscopy detects fluorescence signal (400×). EGFP-*survivin*, exogenous expression; original *survivin*, intracellular endogenous expression.

3.3. p53 and Ki67 gene expression changes under survivin gene overexpression detected by Western blot

The result showed over-expression of Survivin up-regulated the level of p53 and Ki67. Quantity one v4.62 software analyzed the gray value of the strip (Tracking Trace) based on light density curves drawn by different electrophoretic bands of, the light density, and then calculating the area under the optical density curve was regard to the quantitative analysis of the electrophoretic bands (Figure 3, Table 4).

3.4. Bioinformatics analysis of survivin, p53 and Ki67 interactions

By searching STRING database (http://string-db.org), survivin, p53 and Ki67 interaction networks were obtained; and MKI67 and TP53 were linked through cell cycle regulatory signaling pathways. 'Experimental evidence' was selected as a search condition, and received a large number of reported literature on the relationship of MKI67 and TP53 [9–11]. TP53 and BIRC5 had interactions in cell apoptosis control, DNA repair and other processes, which participated in the complex cell cycle regulation [12] (Figure 4).

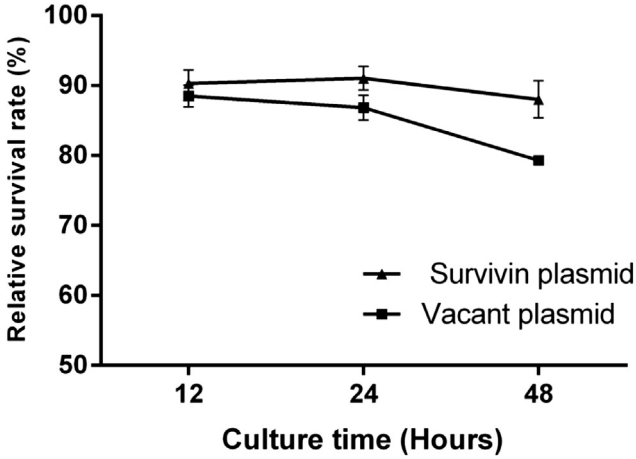

Figure 2. MTT test results.
Relative survival rate standard initial cell absorbance value (490 nm absorbance value).

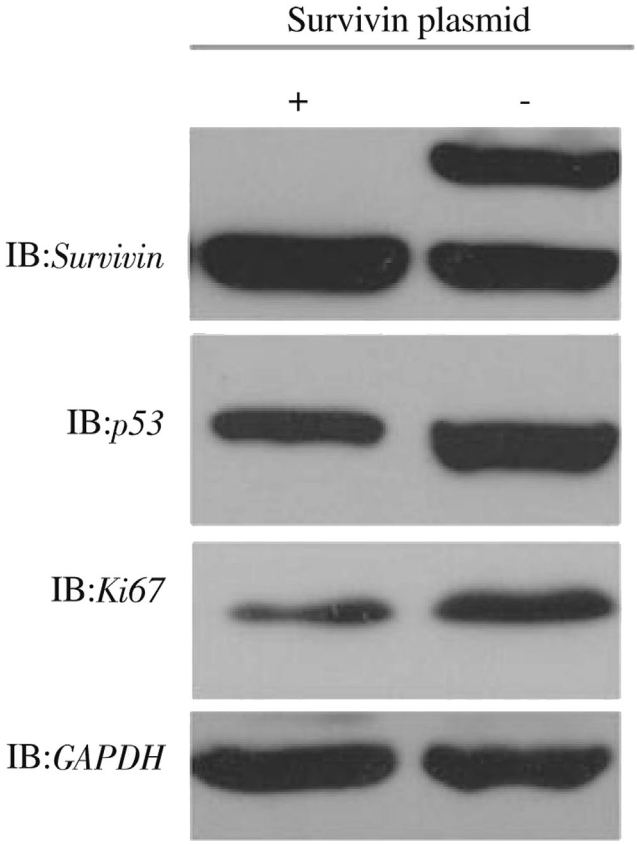

Figure 3. *p53* and *Ki67* gene expression changes under *survivin* gene overexpression.

Table 4
Signal of protein expressions measured by quantity one.

Group[a]	Gene		
	Survivin	*p53*	*Ki67*
+	52.9[b] ± 6.3**	18.2 ± 2.1*	16.2 ± 7.1
–	25.7 ± 3.7	11.7 ± 6.8	10.9 ± 0.9

*$P < 0.05$, **$P < 0.01$.
[a] + Transfected with *survivin* expression plasmid, – Transfected no load control plasmid. [b] Quantity one Trace Tracking automatic calculation of relative expression (that is, target protein band optical density signal, the optical density signal using *GAPDH* for normalization process).

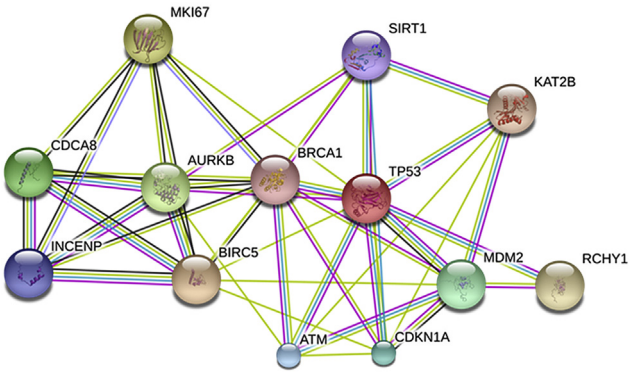

Figure 4. Bioinformatics analysis of survivin, p53 and Ki67 interactions. TP53, tumor protein p53; BIRC5, baculoviral IAP repeat-containing 5; MKI67,antigen identified by monoclonal antibody Ki-67; AURKB, aurora kinase B; CDCA8, cell division cycle associated 8; MDM2, Mdm2 p53 binding protein homolog (mouse); CDKN1A, cyclin-dependentkinase inhibitor 1A (p21, Cip1); ATM, ataxia telangiectasia mutated; INCENP, inner centromere protein antigens 135/155 kDa; SIRT1, sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae); BRCA1, breast cancer 1, early onset; KAT2B, K(lysine) acetyltransferase 2B; RCHY1, ring finger and CHY zinc finger domain containing 1.

3.5. Transwell invasion assay

As shown in Figure 5, stained Hep-2 cells reached the lower chamber (Figure 5A); while the number of cells that reached the bottom of the chamber for Hep-2 cells transfected with empty vector (Figure 5B) and Hep-2 cells transfected with survivin expression plasmid (Figure 5C).

3.6. Correlation analysis of Ki67, p53 and survivin expression in laryngeal cancer

Using rank times the size of a linear variable for *survivin*, *Ki67*, *p53* expressions in laryngeal carcinoma, correlation analysis showed that the three expression in laryngeal carcinoma were positively correlated (r = 0.607, 0.541, 0.648; $P < 0.01$) (Table 5).

4. Discussion

Cancer gene activation, tumor suppressor gene inactivation as well as overexpression of anti-apoptotic genes are important causes of cancer occurrence; and cancer gene expressions results from the regulation of multiple complex factors. Multiple gene cross-talking directly or indirectly leads to tumor occurrence. Thus, tumor occurrence is usually not a single gene effect, but rather results of the interaction of multiple genes, which presents a challenge in tumor development studies.

Cancer gene activation and tumor suppressor gene inactivation under simultaneous action cause cells to lose their ability to regulate their growth; that is, apoptosis regulation disorder, thereby, cells does not undergo normal apoptosis. The significance of apoptosis lies in its ability to maintain cellular

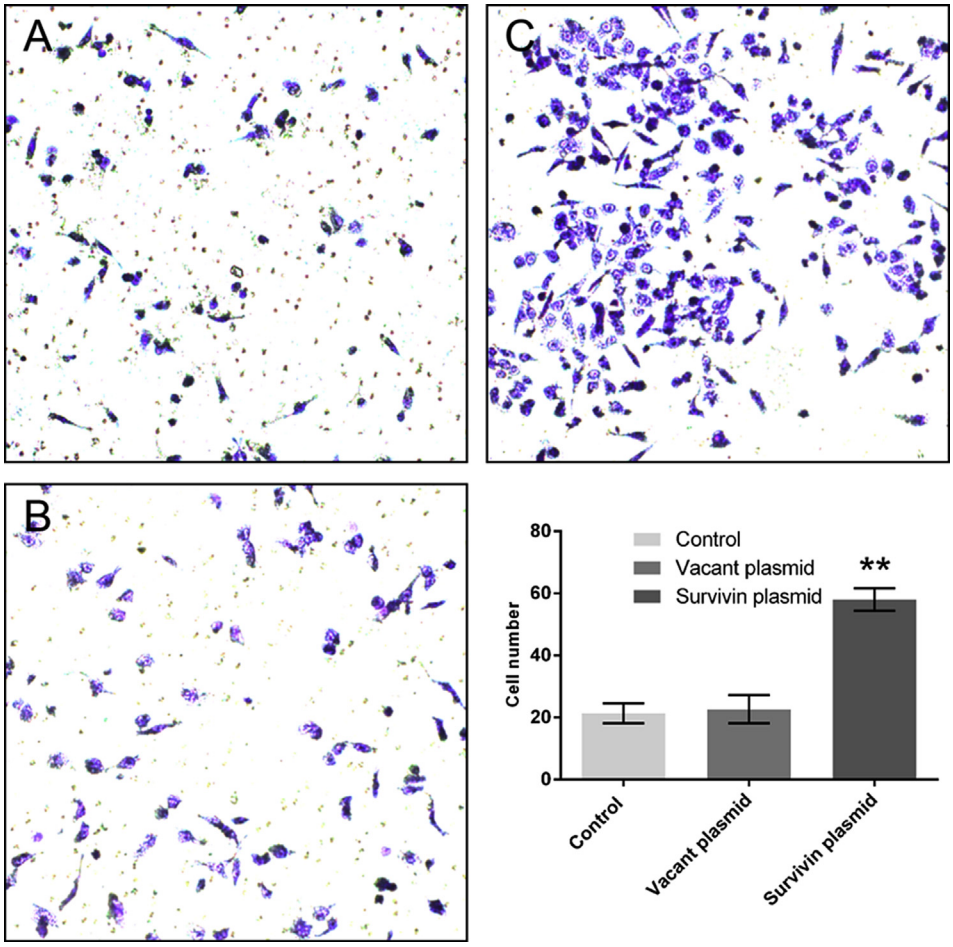

Figure 5. Cell invasion assay.
**: $P < 0.01$.

Table 5
Correlation of *Ki67*, *p53*, *survivin* in laryngeal cancer.

	Survivin				*p53*			
	+	−	*r*	*P*	+	−	*r*	*P*
Ki67								
+	34	8			38	4		
−	4	18	0.607	<0.01	6	16	0.648	<0.01
p53								
+	34	10						
−	4	16	0.541	<0.01				

homeostasis, and it is a kind of genetically controlled programmed cell death. Apoptosis is a physiologically active process that involves a series of gene activation, expression, regulation, as well as other roles. Normal apoptosis is a life-sustaining body necessary for normal metabolism. Once apoptosis-regulatory gene structure or regulatory regions mutate, that is, cells does not undergo normal apoptosis, these cells would ultimately develop into tumors.

Survivin is a member of the inhibitors of apoptosis protein family. Survivin may inhibit apoptosis in two ways: direct inhibition of Caspase-3/7 (apoptosis terminal effector enzyme) activity, and negative regulation of cell apoptosis induced by various stimulation processes. Survivin with cyclin kinase CDK4/2 can block the apoptosis signaling transduction pathway. Survivin can act directly on Caspase by inhibiting Caspasc-3/7 activity, and can also indirectly inhibit Caspase through P21 interactions. Studies have shown that Survivin with cell cycle regulation factors by CDK4 interactions lead to CDK2/cyclin- E activation and ribosomal phosphorylation. Ribosomal phosphorylation rapidly starts up cells to enter the cycle and accelerates the conversion of the G1/S phase, causing P21 released from the Survivin-CDK4 complex combined with mitochondrial pro-caspase-3 to inhibit caspase-3 activity, and prevent mitochondrial release of cytochrome C; and thereby inhibiting cell apoptosis [5]. Studies have also shown that survivin can inhibit the Bax- and Fas-mediated apoptosis pathway [13–15].

p53 is a tumor suppressor gene, and more than 50% of malignancies in clinic appear as mutated genes. The *p53* gene, as cell cycle regulators, can determine the degree of DNA mutation in P53 cells. If DNA damage and mutations are small, it prompts the cell to repair itself; and if DNA damage and mutations are great, p53 induces apoptosis [16]. For this reason, *p53* gene mutations can cause cells not to undergo normal apoptosis, and develop into cancer. It is also one of the causes of p53 mutation expressions in tumor cells.

Studies have shown that wild-type p53 can inhibit the expression of survivin at the transcriptional level, and thereby regulate normal cell cycle and apoptosis. This suggests that the interaction of p53and survivin has an important role for the normal start-up of cell apoptosis [17–19].

In our experiments, we found that the expression of p53 with survivin in nasopharyngeal carcinoma was significantly higher than the adjacent controls, and at the same time, it was also highly expressed in Hep-2 cells; and the expression of these two proteins were highly associated. In survivin overexpression conditions, p53 also appeared to increase. This prompts us that p53 mutation is an important reason for apoptotic cells to lose their normal control; and due to p53 mutations, they lost their ability to inhibit survivin, and further hindered normal apoptosis, thereby causing cells to become cancerous. It also shows that survivin can inhibit apoptosis and cause cell proliferation. Thus, the dual role of inhibiting apoptosis and promoting cell proliferation, in turn, promotes tumor development. To investigate the further coordinating role of p53 and survivin in tumor development, our future research would be transfection of *p53* plasmid gene mutation expressions, as well as studies on the expression and localization changes of survivin, and at the same time, studies on p53 mutation sites (P53 common mutations: aa129–146, 171–179, 234–260, 270–287) with survivin interactions.

Conflict of interest statement

We declare that we have no conflict of interest.

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