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Laryngeal squamous cell carcinoma progression is associated with the NFκB signaling pathway regulated by IκB kinase β

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Abstract. – OBJECTIVE: We aimed to analyze the association of NFκB signaling pathway with the carcinogenesis of laryngeal squamous cell carcinoma (LSCC).

PATIENTS AND METHODS: Protein array was used to identify the differentially expressed proteins involved in NFκB signaling pathway between LSCC cells and normal throat mucosa. Correlation analysis between significantly expressed proteins and clinical characteristics (differentiation, clinical stage, and node metastasis) was performed. The expression of IκB kinase β (IKK-β) in Hep-2 cells transfected with IKK-β-siRNA or pcDNA3.1-IKK-β was detected both by real-time polymerase chain reaction and western blot. Besides, a tetrazolium-based colorimetric assay (MTT), flow cytometer, and transwell assay were used to examine the proliferation, apoptosis, and migration rate of Hep-2 cells, respectively.

RESULTS: Three differentially expressed proteins were identified. Among them, tumor necrosis factor receptor 1 (TNFR1) and IKK-β were significantly up-regulated ($p < 0.01$) and Fas-associated via death domain (FADD) was significantly down-regulated ($p < 0.01$). The correlation analysis showed that IKK-β expression had significant association with differentiation, clinical stage, and node metastasis ($p < 0.05$). Besides, high expressed IKK-β resulted in significantly increased proliferation and migration rate ($p < 0.05$). Reversely, Hep-2 cells transfected with IKK-β-siRNA showed significantly lower proliferation and migration rate ($p < 0.05$) and significantly higher apoptosis rate ($p < 0.05$) than normal cells.

CONCLUSIONS: The NFκB signaling pathway involved TNFR1, IKK-β, and FADD is significantly associated with the development of LSCC. Over-expressed IKK-β efficiently inhibits cell apoptosis and has positive effects on cell proliferation and migration.

Key Words:
Laryngeal squamous cell carcinoma, Progression, NFκB signaling pathway, IKK-β, siRNA.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most common malignant tumors of head and neck[1]. The incidence rate is high in Southeast Asia and Eastern Europe, and remains high around the world[2,3]. In LSCC, the various tumor growth rate is an important factor for survival[4]. In the early stage, LSCC is often curable with surgery or radiotherapy, and nuclear factor-κB (NFκB) has been suggested as a useful marker of radioresistance and prognosis[5-7].

NFκB is constitutively deregulated in several human cancers, including pancreas, breast, liver, stomach, and head and neck squamous cell carcinoma[8-11]. Its activation speeds cancer cells proliferation, inhibits apoptosis, promotes angiogenesis, and induces epithelial-mesenchymal transition[12,13]. Previous study has shown that the nuclear localization of NFκB also plays an important role in the development of LSCC[14]. Huang et al[7] have found that NFκB expression was significantly higher in LSCC cells than in normal tissues and tended to positively associate with lymph node metastasis. It has been suggested that patients with low expression of NFκB had longer overall survival time than those with high NFκB expression[15]. Besides, Katarzyna et al[16] found that NFκB pathway molecules were significantly expressed and had a strong relationship with the aggressiveness of LSCC cells. For example, Zhang et al[17] found that activation of NFκB transcriptional activity by S100A4 was contributed to the increasing of matrix metalloproteinase-9, which promoted the invasion of LSCC cell line Hep-2. Although several studies have focused on the relationship between NFκB pathway and the carcinogenesis of LSCC, the details remain unclear[5,7,18].

In the present study, we used protein array to analyze the differentially expressed proteins re-

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Table I. Chips map of protein array.

1	2	3	4	5	6	7	8
Pos1	Pos2	Pos3	Neg	TNF-a	TNF-b	MKP	PLA_2
Pos1	Pos2	Pos3	Neg	TNF-a	TNF-b	MKP	PLA_2
TNFR1	TRAF2	RIP	FADD	P50	JNK	SOD2	TRADD
TNFR1	TRAF2	RIP	FADD	P50	JNK	SOD2	TRADD
RelB	IKK-α	IKK-β	p52	ROS	Caspase8	C-Rel	Pos1
RelB	IKK-α	IKK-β	p52	ROS	Caspase8	C-Rel	Pos1

Pos: positive; Neg: negative.

Table II. The correlation analysis between differentially expressed proteins and clinicopathologic characteristics.

Clinicopathologic characteristics	Cases	TNFR1	*p*-value	IKK-β	*p*-value	FADD	*p*-value
Differentiation							
Well	24	2464.23	0.254	1775.48	0.021	286.34	0.152
Poorly	26	2632.56		3482.31		322.45	
TNM classification							
Grade I and II	16	2782.35	0.287	1834.42	0.028	269.34	0.234
Grade III and IV	34	2490.32		3452.95		315.68	
Node metastasis							
Without	38	2895.53	0.358	1874.25	0.035	294.68	0.138
With	12	2423.64		3449.58		324.52	

$p < 0.05$ was considered to be significantly different.

lated to NFκB signaling pathway in LSCC compared with normal tissues. Effects of the most differentially expressed protein on proliferation, apoptosis, and migration were analyzed using over-expression or RNA interfering method, and the results were detected by a tetrazolium-based colorimetric assay (MTT), flow cytometer, and transwell assay, respectively.

Patients and Methods

Clinicopathologic Characteristics of Patients

The LSCC cases and normal tissues were recruited from the Department of Otorhinolaryngology at the Second peoples' hospital in Shanghai, China. A total of 50 LSCC patients (aged 40-62 years, average 54 years; gender ratio 1: 1) confirmed by histopathological examination were included in this study. Among them, 38 patients had node metastasis. According to a differential degree, 24 cases were well-differentiated and 26 poorly-differentiated. Tumors were histologically graded to 4 stages (I: 5 cases, II: 11 cases, III: 28 cases, IV: 6 cases) based on the tumor node metastasis (TNM) classification of the International Union Against Cancer (UICC). In addition, the normal throat mucosal tissues collected from 50 cases (aged 43-60 years, average 52 years; gender ratio 1: 1) were considered as control. All studies were approved by the ethics committee of the Second peoples' hospital in Shanghai, China, and all participants had given their informed content.

Protein Array

LSCC and control tissues were lysed by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China), and the total protein concentration in the supernatant was detected using the bicinchoninic acid (BCA) method (BCA protein assay kit, Raybiotech, Norcross, GA, USA). The procedures were performed strictly as described in the instruction. Control and tumor samples were divided into two equal portions. After drying at room temperature for 2 h, 100 μl blocking solution was added to each column, and the slides were incubated for 1 h under shaking. After incubation, blocking solution was discard-

ed. Tissue lysate was diluted to a final concentration of 500 μg/ml and was added to the samples, and then incubated at 4°C overnight. After washed, CY3-labeled antibody mixture was added and incubated at room temperature for 2 h, then the samples were washed again and scanned using GenePix4000B (Molecular Devices, Sunnyvale, CA, USA).

Construction of Eukaryotic Expression Vector

Total RNA was extracted using Trizol (Gibco-BRL, Rockville, MD, USA). Oligo-dT, dNTP, DTT, and reverse transcriptase were used in the synthesis of cDNA. The sequence of IκB kinase (IKK-β) was amplified from 5 μl cDNA template via PCR using special primers. Forward: 5' CCAAGCTTCCACCATGAGCTGGTCAC 3'. Reverse: 5' GGGGTACCTGAGGCCTGCTCCAGGC 3'. The resultant PCR fragment was purified with gel extraction kit (Qiagen, Valencia, CA, USA), treated with *HindIII* and *KpnI* and purified again. Then, the restricted IKK-β fragment was inserted into vector pcDNA3.1 at the same digestion sites to construct pcDNA3.1-IKK-β, which was verified by PCR and sequencing.

SiRNA Preparation

SiRNA was designed according to IKK-β mRNA sequence applied in Human Genedatabase using Ambion software, and was synthesized by Borui Biology (Guangzhou, China). The target sequence was CCAATAATCTTAACAGTGT. The sense strand of siRNA was 5' CCAAUAAUCUUAACAGUGUdTdT 3'. Anti-sense: 3' dTdTGGUUAUUAGAAUUGUCACA 5'. Non-targeted siRNAs were designed as negative control.

Transfection of Hep-2 cells

Hep-2 cells obtained from the Chinese academy of science (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2 overnight. Hep-2 cells were separately transfected with IKK-β-siRNA, pcDNA3.1-IKK-β, pcDNA3.1, or negative control siRNA according to the manufacturer's instructions (HiPerFect transfection reagent, Qiagen, Inc., Valencia, CA, USA). Hep-2 cells (2×10^5 cells/ml) were plated into independent wells of 6-well (2 ml) and 96-well dish (100 μl). Then, 3 μl HiPerFect transfection reagents were added and mixed gently. After incubated for 10 min at room temperature, cells mixture was slowly added to the wells at 37°C under 5% CO_2 for 6 h. Subsequently, transfection reagent was replaced by DMEM medium and incubated for 24 h, 48 h, and 72 h, respectively.

Real-time Polymerase Chain Reaction (RT-PCR) and Western Blot

Total RNA was extracted from cells incubating separately for 24 h, 28 h, and 72 h as described above. RNA was quantified and cDNA was synthesized. The primers for IKK-β were 5' GACTTGAATGGAACGGTGAA 3' (Forward) and 5' TCTTGGGCTCTTGAAGGATA 3' (Reverse). The primers for β-actin were 5' CTCCCACCTTATCTACTCCC 3' (Forward) and 5' TAGCTGCTCGCTGTCTTG 3' (Reverse). Amplification conditions: 94°C, 5 min; 95°C, 30 s; 55°C, 30 s; 72°C, 30 cycles of 30 s; 72°C, 10 min. The PCR products were isolated by agarose gel electrophoresis and quantified by a Gel imager. β-actin was used as an internal control.

Cells were lysed and total protein concentration was detected. A total of 50 μl lysate was sampled to SDS-PAGE. After electrophoresis, the separated target proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blotted with 5% non-fat dry milk. Then, the membrane was incubated overnight with anti-IKK-β or anti-β-actin (Abcam, Cambridg, UK). After incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody (IgG, Abcam, Cambridge, UK) and washing, the protein bands were detected with chemiluminescence.

MTT Assay

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the viability of Hep-2 cells cultured for 24 h, 48 h, and 72 h, respectively. Cells grown in 96-well multiplates were added with 20 μl MTT (5 mg/mL) and incubated for 4 h. Then, the supernant was discarded and 150 μl dimethyl sulfoxide (DMSO) was added. After incubation for 10 min with shaking, the final product was quantified spectrophotometrically by absorbance at 570 nm wavelength.

Flow Cytometry

Cellular apoptosis was assessed by flow cytometry. Trypsinized cells were collected at 2000 rpm × 10 min and washed 3 times with phosphate-buffered saline (PBS). Propidium iodide (PI) was added and the cells were incubated for 15 min at 4°C in the dark. The stained cells were

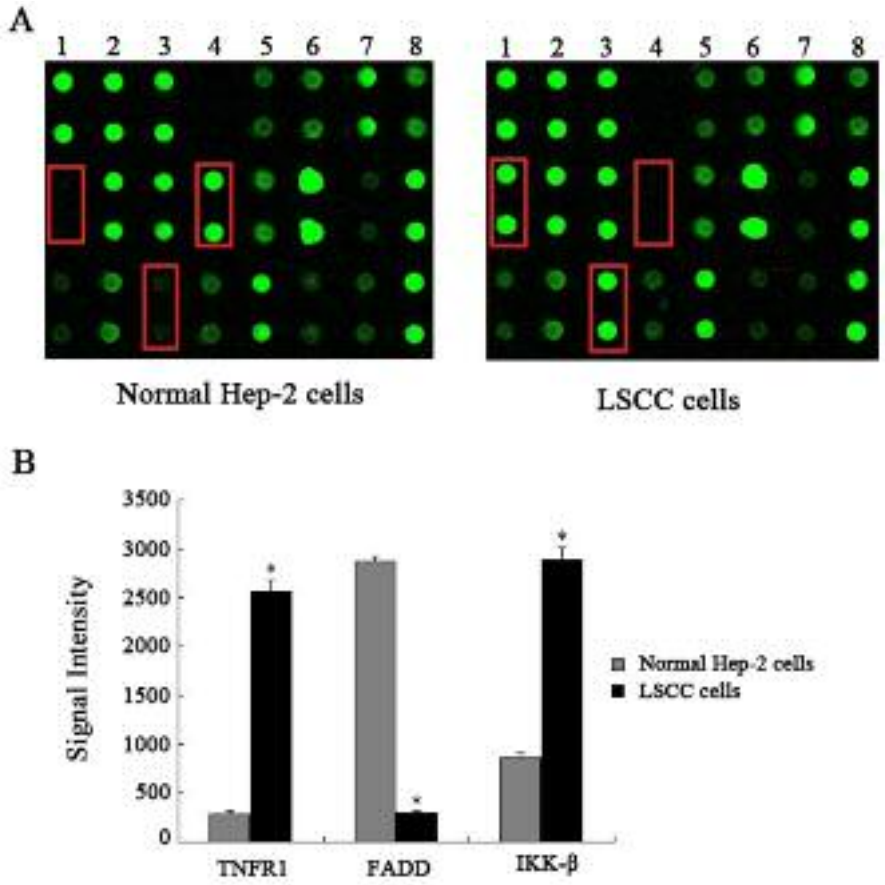

Figure 1. Identification of differentially expressed proteins in LSCC cells. Each sample was performed in duplicate. ***A,*** Protein array; Red rectangles represent differentially expressed proteins. ***B,*** Quantifications of differentially expressed proteins. $^{*}p < 0.05$.

analyzed by Elite Esp flow cytometer (Beckman Coulter, Miami, FL, USA).

Transwell Migration Assay

For cell migration assay, 5 $\times 10^5$ cells were placed in the upper chamber, and 500 μl medium without fetal bovine serum was added to the lower chamber. An 8 μm polycarbonate membrane was placed between the two champers. After migration for 24 h at 37°C under 5% CO_2, cells in the upper chamber of transwell filter were removed with a cotton swab. Then, the membrane was fixed in methanol for 10 min, stained with Giemsa for 20 min. The migration number was counted in 5, 400× microscopic fields per filter.

Statistical Analysis

Statistical analysis was performed using SPSS12.0 software (SPSS Inc., Chicago, IL, USA). Comparisons between two groups were accomplished using *t* test, and correlation analyses between differentially expressed proteins and clinicopathologic characteristics were conducted by Sperman analysis. $p < 0.05$ was considered to be statistically significant.

Results

TNFR1, IKK-β and FADD were Differentially Expressed in LSCC Cells

Chips map of protein array was displayed in Table I. Protein array showed that both tumor necrosis factor receptor 1 (TNFR1) and IKK-β were significantly over-expressed, while Fas-associated via death domain (FADD) was significantly down-expressed in LSCC cells ($p < 0.01$, Figure 1).

The correlation between the differentially expressed cytokines and clinicopathological characteristics was shown in Table II. The signal value of IKK-β expression was significantly higher

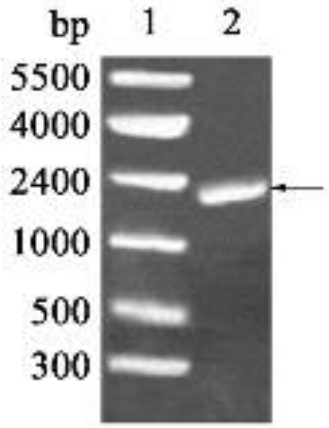

Figure 2. PCR analysis for plasmid pcDNA3.1-IKK-β. 1: Marker; 2: pcDNA3.1-IKK-β.

in poorly-differentiated tissues than well-differentiated tissues (3482.31 vs. 1775.48, $p < 0.05$), and higher in clinical grade III and IV tissues than clinical grade I and II tissues (3452.95 vs. 1834.42, $p < 0.05$). Moreover, it was also significantly increased in tissues with node metastasis than those without node metastasis (3449.58 vs. 1874.25, $p < 0.05$). The correlations between the other two cytokines and clinicopathological characteristics were not significant ($p > 0.05$).

Construction of pcDNA3.1-IKK-β Expression Plasmid

After the pcDNA3.1-IKK-β expression plasmid was amplified by PCR, a 2270 bp fragment was obtained (Figure 2). The length of the fragment was consistent with IKK-β gene, and the sequencing result was also the same as reported in GenBank.

The Expression of IKK-β in Hep-2 Cells Transfected with pcDNA3.1-IKK-β or IKK-β-siRNA

An approximately 520 bp amplified fragment of IKK-β was observed after semiquantitative RT-PCR (Figure 3). After IKK-β-siRNA transfected cells incubated for 24 h, 48 h, or 72 h, the expression of IKK-β was obviously down-regulated (Figure 3A). In addition, after transfected with pcDNA3.1-IKK-β, IKK-β expression was significantly up-regulated (Figure 3B). Western blot showed the same results (Figure 3C, D). The expression of IKK-β was not changed in negative control and control cells.

Proliferation, Apoptosis, and Migration rate in Hep-2 Cells Transfected with pcDNA3.1-IKK-β or IKK-β-siRNA

As shown in Figure 4A, the proliferation rate of negative control or pcDNA3.1 transfected cells was not changed at 24 h, 48 h, or 72 h ($p > 0.05$). Besides, the proliferation rate of IKK-β-siRNA transfected cells was significantly decreased ($p < 0.05$), and the proliferation rate was significantly increased ($p < 0.05$) in cells transfected with pcDNA3.1-IKK-β. For example, the proliferation rate of pcDNA3.1-IKK-β transfected cells increased from 145% (24 h) to 250% (72 h).

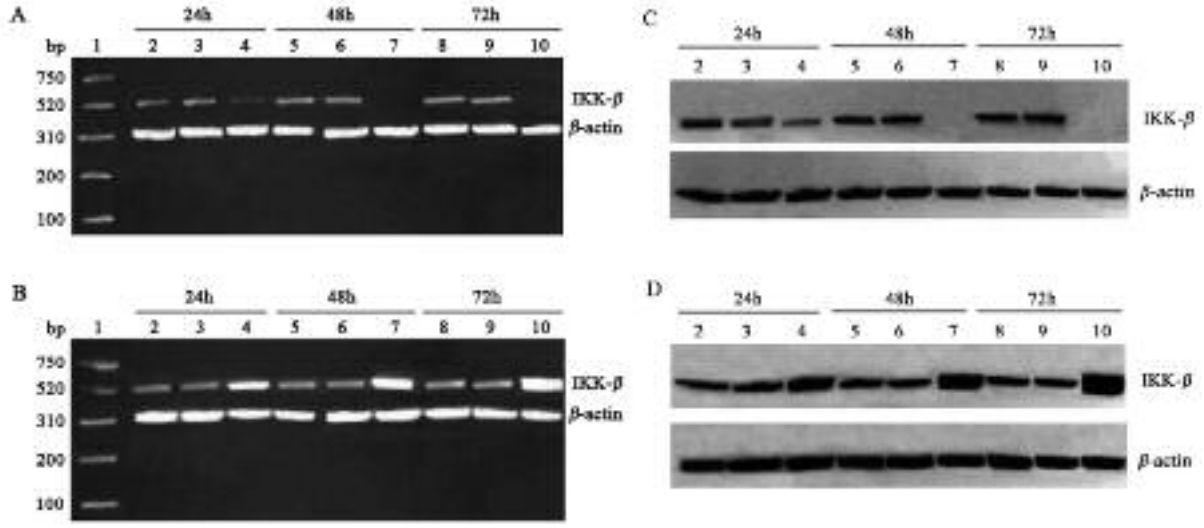

Figure 3. RT-PCR and western blot. ***A-C***: 1: Marker; 2, 5, 8: controls; 3, 6, 9: non-targeted siRNA; 4, 7, 10: IKK-β siRNA. ***B-D***: 3, 6, 9: pcDNA3. 1; 4, 7, 10: pcDNA3.1-IKK-β.

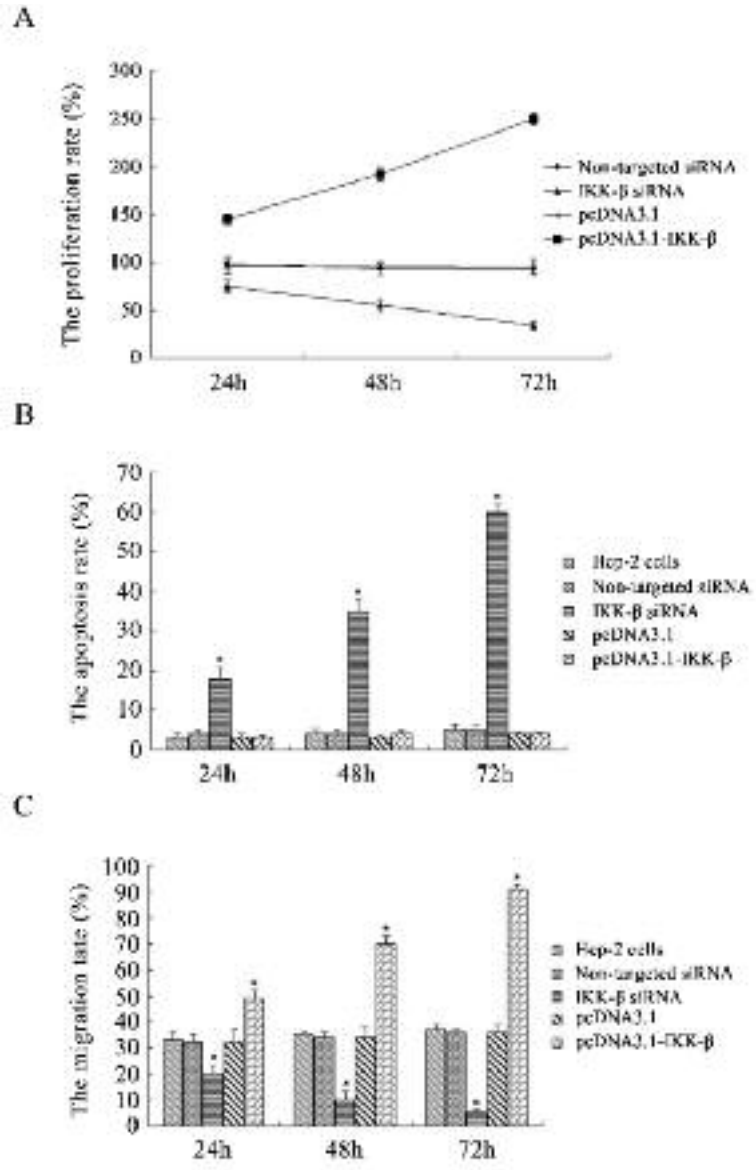

Figure 4. Proliferation ***(A)***, apoptosis ***(B)***, and migration analysis ***(C)*** in cells transfected with IKK-β siRNA, non-targeted siRNA, and pcDNA3.1-IKK-β. $^{*}p < 0.05$.

The apoptosis rate was similar among negative controls, pcDNA3.1-IKK-β transfected cells, pcDNA3.1 transfected cells, and control cells. Besides, the apoptosis rate of IKK-β-siRNA transfected cells was significantly increased (from 18% at 24 h to 60% at 72 h) compared with control cells ($p < 0.05$, Figure 4B).

Cells migration rate did not differ in negative control and pcDNA3.1 transfected cells compared to control cells ($p > 0.05$, Figure 4C). However, it was significantly reduced (from 20% at 24 h to 5% at 72 h) in IKK-β-siRNA transfected cells ($p < 0.05$), and was significantly increased (from 49% at 24 h to 91% at 72 h) in pcDNA3.1-IKK-β transfected cells compared with control ($p < 0.05$).

Discussion

The development and migration of LSCC is a complex process which is associated with changeable expression of multiple genes[19-22]. In the present study, 3 differentially expressed cy-

tokines (TNFR1, IKK-β, and FADD) involved in NFκB signaling pathway were identified in LSCC cells compared with controls using protein array. Among them, IKK-β had significant correlation with clinicopathological characteristics. Furthermore, we found that IKK-β expression had significant effects on proliferation, apoptosis, and migration of Hep-2 cells.

TNFR1, a type I transmembrane glycoprotein, is one of the TNF-α homologous receptors. It is responsible for many biological processes of TNF, such as programmed cell death, antiviral activity, and activation of NFκB[23]. The activation of NFκB or ligation with its adapter proteins including TNF receptor-associated protein with a death domain (TRADD) and serine-threonine kinase receptor-interacting protein 1 (RIP1) is carried out through a death domain of TNFR1[24]. Once associated with TNFR1, TRADD would recruit TRAF2, which forms complexity with cellular inhibitor of apoptosis protein (cIAP) -1 and cIAP-2 via its N-terminal RING domain[25,26]. Then, the formed complex recruits TGF-β activated kinase (TAK) 1 and IKK proteins[27] and subsequently activates the anti-apoptotic pathway mediated by NFκB. Although NFκB signaling pathway can be activated by different signals, the final step is mediated by IKK proteins. IKK proteins consist of IKK-α, IKK-β, and IKK-γ. Among them, IKK-β is the most important catalytic subunit and its active effect is an early event in the activation of NFκB pathway[28,29]. Previous studies have suggested that the absence of IKK-β will block the activation of NFκB through protecting the inhibitory subunit IκBα from degradation, and thereby inhibit cell growth[30-32]. Besides, FADD is a common conduit in TNF-α mediated apoptosis. In this process, a death inducing signaling complex comprised of TRADD, FADD, and pro-caspase-8 is formed, and subsequently initiates a cascade downstream apoptotic signaling including BH3 interacting domain death agonist, Cytochrome c, Apaf-1, caspase-9, and caspase-3, and thereby promotes cell apoptosis[33,34]. In our present study, TNFR1 and IKK-β were high-expressed and FADD was down-expressed in LSCC cells. Therefore, we suggest that abnormal expressions of TNFR1, IKK-β, and FADD are closely associated with the activation of NFκB signaling pathway. In the development of LSCC, both apoptotic pathway involved FADD and anti-apoptotic pathway mediated by IKK-β are initiated in LSCC cells. However, the anti-apoptotic pathway is dominant due to high expression of IKK-β and low expression of FADD.

Additionally, we found that the expression level of IKK-β is significantly correlated with poorly-differentiation, high histologic grade and node metastasis in our study. Tang et al[35] reported that activation of genes along NFκB pathway including NIK, IKK-α, IKK-β, and NFκB gene were important in initiating cell proliferation. In mice, IKK-β-deficient embryonic fibroblasts died at approximately 14.5 days of gestation because of liver degeneration and apoptosis[36]. Hence, it has been indicted that suppression of the activation of NFκB signaling pathway can lead to enhanced apoptosis of tumor cells[31,37]. Besides, in gliomas, inactivation of NFκB pathway by down-regulating IKK-β markedly reduced the migration and invasion ability of cells[38]. In highly-metastatic human breast cancer cells, induced apoptotic effect and suppressed migration and invasion were also found because of decreased expression of several genes including IKK-α and IKK-β[39]. Our research indicates a similar result if compared to the previous studies. Thus, abnormal IKK-β expression has significant effects on proliferation, apoptosis, and migration of Hep-2 cells through association with the NFκB signaling pathway in LSCC cells.

Conclusions

Overall, the NFκB signaling pathway related to TNFR1, IKK-β, and FADD is significantly associated with the development of LSCC. Over-expressed IKK-β has positive effects on cells proliferation and migration, and efficiently inhibits apoptosis. However, this is a multiplex process since there are several chains combining TNFR1 with stimulation of IKK complex and activation of the classical NFκB signaling pathway. Therefore, further knockout or point mutation experiments for one gene or multiple genes involved in NFκB signaling pathway are still needed to further study the details in the carcinogenesis of LSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Chu EA, Kim YJ. Laryngeal cancer: diagnosis and preoperative work-up. Otolaryng Clin N Am 2008; 41: 673-695.

2) Robin P, Reid A, Powell D, Mcconkey C. The incidence of cancer of the larynx. Clin Otolaryngol Allied Sci 1991; 16: 198-201.

3) Braakhuis BJ, Leemans CR, Visser O. Incidence and survival trends of head and neck squamous cell carcinoma in the Netherlands between 1989 and 2011. Oral Oncol 2014; 50: 670-675.

4) Van Bockel LW, Verduijn GM, Monninkhof EM, Pameijer FA, Terhaard CH. The importance of actual tumor growth rate on disease free survival and overall survival in laryngeal squamous cell carcinoma. Radiother Oncol 2014; 112: 119-124.

5) Yoshida K, Sasaki R, Nishimura H, Okamoto Y, Suzuki Y, Kawabe T, Saito M, Otsuki N, Hayashi Y, Soejima T, Nibu K, Sugimura K. Nuclear factor-□B expression as a novel marker of radioresistance in early-stage laryngeal cancer. Head Neck 2010; 32: 646-655.

6) Jiang LZ, Wang P, Deng B, Huang C, Tang WX, Lu HY, Chen HY. Overexpression of Forkhead Box M1 transcription factor and nuclear factor-□B in laryngeal squamous cell carcinoma: a potential indicator for poor prognosis. Hum Pathol 2011; 42: 1185-1193.

7) Huang C, Huang K, Wang C, Jiang ZD, Li XX, Wang HP, Chen HY. Overexpression of mitogen-activated protein kinase kinase 4 and nuclear factor-kappaB in laryngeal squamous cell carcinoma: a potential indicator for poor prognosis. Oncol Rep 2009; 22: 89-95.

8) Lin SC, Liu CJ, Yeh WI, Lui MT, Chang KW, Chang CS. Functional polymorphism in NF□B 1 promoter is related to the risks of oral squamous cell carcinoma occurring on older male areca (betel) chewers. Cancer Lett 2006; 243: 47-54.

9) Hayden MS, Ghosh S. Shared principles in NF-κB signaling. Cell 2008; 132: 344-362.

10) Sethi G, Sung B, Aggarwal BB. Nuclear factor-κB activation: from bench to bedside. Exp Biol Med 2008; 233: 21-31.

11) Allen CT, Ricker JL, Chen Z, Van Waes C. Role of activated nuclear factor-κB in the pathogenesis and therapy of squamous cell carcinoma of the head and neck. Head Neck 2007; 29: 959-971.

12) Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu Rev Immunol 2000; 18: 621-663.

13) Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. J Clin Invest 2001; 107: 241-246.

14) Du J, Chen GG, Vlantis AC, Xu H, Tsang RK, Van Hasselt AC. The nuclear localization of NF□B and p53 is positively correlated with HPV16 E7 level in laryngeal squamous cell carcinoma. J Histochem Cytochem 2003; 51: 533-539.

15) Starska K, Kulig A, Łukomski M. Tumor front grading in prediction of survival and lymph node metastases in patients with laryngeal carcinoma. Adv Med Sci 2006; 51: 200-204.

16) Starska K, Forma E, Lewy-Trenda I, Stasikowska O, Bry M, Krajewska WM, Łukomski M. The expression of SOCS1 and TLR4-NFkappaB pathway molecules in neoplastic cells as potential biomarker for the aggressive tumor phenotype in laryngeal carcinoma. Folia Histochem Cytobiol 2010; 47: 401-400.

17) Zhang W, Liu Y, Wang C. S100A4 promotes squamous cell laryngeal cancer Hep-2 cell invasion via NF-kB/MMP-9 signal. Eur Rev Med Pharmacol Sci 2014; 18: 1361-1367.

18) Anto RJ, Mukhopadhyay A, Shishodia S, Gairola CG, Aggarwal BB. Cigarette smoke condensate activates nuclear transcription factor-κB through phosphorylation and degradation of IκBα: correlation with induction of cyclooxygenase-2. Carcinogenesis 2002; 23: 1511-1518.

19) Spafford MF, Koeppe J, Pan Z, Archer PG, Meyers AD, Franklin WA. Correlation of tumor markers p53, bcl-2, CD34, CD44H, CD44v6, and Ki-67 with survival and metastasis in laryngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 1996; 122: 627-632.

20) Zhang B, Liu W, Li L, Lu J, Liu M, Sun Y, Jin D. KAI1/CD82 and CyclinD1 as biomarkers of invasion, metastasis and prognosis of laryngeal squamous cell carcinoma. Int J Clin Exp Pathol 2013; 6: 1060-1067.

21) Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med 1995; 332: 429-435.

22) Jin YT, Kayser S, Kemp BL, Ordonez NG, Tucker SL, Clayman GL, Goepfert H, Luna MA, Batsakis JG, El-Naggar AK. The prognostic significance of the biomarkers p21WAF1/CIP1, p53, and bcl-2 in laryngeal squamous cell carcinoma. Cancer 1998; 82: 2159-2165.

23) Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell 1995; 81: 495-504.

24) Chan FK. Three is better than one: pre-ligand receptor assembly in the regulation of TNF receptor signaling. Cytokine 2007; 37: 101-107.

25) Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003; 10: 45-65.

26) Zheng C, Kabaleeswaran V, Wang Y, Cheng G, Wu H. Crystal structures of the TRAF2: cIAP2 and the TRAF1: TRAF2: cIAP2 complexes: affinity, specificity, and regulation. Mol Cell 2010; 38: 101-113.

27) Jackson-Bernitsas D, Ichikawa H, Takada Y, Myers J, Lin X, Darnay B, Chaturvedi M, Aggarwal B. Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-kappaB activation and proliferation in human head and neck squamous cell carcinoma. Oncogene 2006; 26: 1385-1397.

28) Lim SY, Song YJ. The extract of Tamarindus indica L. suppresses IKKβ activity and NF-κB-dependent lymphoblastoid cell line survival. Mol Cell Toxicol 2013; 9: 243-248.

29) Kim SG, Veena MS, Basak SK, Han E, Tajima T, Gjertson DW, Starr J, Eidelman O, Pollard HB, Srivastava M, Srivatsan ES, Wang MB. Curcumin treatment suppresses IKKβ kinase activity of salivary cells of patients with head and neck cancer: a pilot study. Clin Cancer Res 2011; 17: 5953-5961.

30) Wu DG, Yu P, Li JW, Jiang P, Sun J, Wang HZ, Zhang LD, Wen MB, Bie P. Apigenin potentiates the growth inhibitory effects by IKK-β-mediated NF-κB activation in pancreatic cancer cells. Toxicol Lett 2014; 224: 157-164.

31) Li HP, Zeng XC, Zhang B, Long JT, Zhou B, Tan GS, Zeng WX, Chen W, Yang JY. miR-451 inhibits cell proliferation in human hepatocellular carcinoma through direct suppression of IKK-β. Carcinogenesis 2013; 34: 2443-2451.

32) Vlantis AC, Lo CS, Chen GG, Ci Liang N, Lui VW, Wu K, Deng YF, Gong X, Lu Y, Tong MC, van Hasselt CA. Induction of laryngeal cancer cell death by Ent-11-hydroxy-15-oxo-kaur-16-en-19-oic acid. Head Neck 2010; 32: 1506-1518.

33) Jones BE, Lo CR, Liu H, Srinivasan A, Streetz K, Valentino KL, Czaja MJ. Hepatocytes sensitized to tumor necrosis factor-α cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. J Biol Chem 2000; 275: 705-712.

34) Zhang X, Vallabhaneni R, Loughran PA, Shapiro R, Yin XM, Yuan Y, Billiar TR. Changes in FADD levels, distribution, and phosphorylation in TNFα-induced apoptosis in hepatocytes is caspase-3, caspase-8 and BID dependent. Apoptosis 2008; 13: 983-992.

35) Tang JB, Xu Y, Wang XT. Tendon healing in vitro: activation of NIK, IKKalpha, IKKbeta, and NF-kappaB genes in signal pathway and proliferation of tenocytes. Plast Reconstr Surg 2004; 113: 1703-1711.

36) Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, Goeddel DV. Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. Immunity 1999; 10: 421-429.

37) Phromnoi K, Reuter S, Sung B, Prasad S, Kannappan R, Yadav VR, Chanmahasathien W, Limtrakul P, Aggarwal BB. A novel pentamethoxyflavone down-regulates tumor cell survival and proliferative and angiogenic gene products through inhibition of IB kinase activation and sensitizes tumor cells to apoptosis by cytokines and chemotherapeutic agents. Mol Pharmacol 2011; 79: 279-289.

38) Song L, Huang Q, Chen K, Liu L, Lin C, Dai T, Yu C, Wu Z, Li J. miR-218 inhibits the invasive ability of glioma cells by direct downregulation of IKK-. Biochem Bioph Res Co 2010; 402: 135-140.

39) Wang S, Zhong Z, Wan J, Tan W, Wu G, Chen M, Wang Y. Oridonin induces apoptosis, inhibits migration and invasion on highly-metastatic human breast cancer cells. Am J Chinese Med 2013; 41: 177-196.