Original Article

IκB kinase β Mediating the Downregulation of p53 and p21 by Lipopolysaccharide in Human Papillomavirus 16+ Cervical Cancer Cells

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Abstract

Background: Cervical cancer is the second most common cancer of woman in the world, and human papillomavirus (HPV) infection plays an important role in the development of most of the cases. IκB kinase β (IKKβ) is a kinase-mediating nuclear factor kappa B (NF-κB) activation by phosphorylating the inhibitor of NF-κB (IκB) and is related by some diseases caused by virus infection. However, there is little known about the correlation between IKKβ and HPV infection in cervical cancer. This study aimed to investigate the expression of IKKβ protein in cervical cancer tissues and effects of inflammation on HPV positive or negative cervical cancer cells through detecting the expression of IKKβ, IκBα, p53, and p21 proteins after treated with lipopolysaccharide (LPS) to mimic bacterial infection. We also examined the effects of LPS on cervical cancer cells after blocking IKKβ with pharmacological inhibitor.

Methods: Thirty-six matched specimens of cervical cancer and adjacent normal tissues were collected and analyzed in the study. The expression of IKKβ in the tissue specimens was determined by immunohistochemical staining. In addition, Western blot was used to detect the expression level changes of IKKβ, IκBα, p53, and p21 after LPS stimulated in the HPV16$^+$ (SiHa) and HPV16$^-$ (C33A) cervical cancer cell lines. Furthermore, the effects of IKKβ inhibitor SC-514 on LPS-induced expression change of these proteins were investigated.

Results: The expression of IKKβ was higher in cervical cancer than adjacent normal tissues, and there was no significant difference between tumor differentiation, size, and invasive depth with IKKβ expression. The LPS, which increased the expression level of IKKβ protein but decreased in the IκBα, p53 and p21 proteins, was illustrated in HPV16$^+$ (SiHa) but not in HPV16$^-$ (C33A) cells. Moreover, IKKβ inhibitor SC-514 totally reversed the upregulation of IKKβ and downregulation of p53 and p21 by LPS in SiHa cells.

Conclusions: IKKβ may mediate the downregulation of p53 and p21 by LPS in HPV16$^+$ cervical cancer cells.

Key words: Cervical Cancer; IKKβ; Lipopolysaccharide; p21; p53

Introduction

Cervical cancer is the second most common cancer of woman in the world.[1] Human papillomavirus (HPV) infection is involved in the development of 99.8% of cases.[2] Nuclear factor kappa B (NF-κB) as an important transcriptional factor is related with HPV-induced tumorigenesis.[3-5] Activated IκB kinase β (IKKβ) phosphorylates the inhibitor of NF-κB (IκB), which binds NF-κB to inhibit its function, leading to the degradation of IκB and free of NF-κB entering into the nucleus where it activates various target genes.[6-8] IKKβ can also phosphorylate p53, which is a critical tumor suppressor that activates lots of genes including p21, Bax, and Puma at the transcriptional level, promoting its degradation by β-TrCP.[9] Loss of IKKβ activity increases the stability of p53 and expression of p21 resulting in cell cycle arrest and apoptosis.[10] However, there is little known about the function of IKKβ in cervical cancer.

In this study, we investigated the expression of IKKβ protein in cervical cancer tissues and effects of inflammation in HPV

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positive or negative cervical cancer cells through detecting the expression of IKKβ, IκBα, p53, and p21 proteins after treated with lipopolysaccharide (LPS) to mimic bacterial infection. In addition, we also examined the effects of LPS on cervical cancer cells after blocking IKKβ with pharmacological inhibitor.

Methods

Patient recruitment and specimen collection

A total of 36 pairs of formalin-fixed specimens with clinical data were collected from cervical cancer patients at Xiangya Hospital, Central South University. Informed consent was obtained from each patient. The cervical cancer and adjacent normal tissues before surgery were collected in the study. The inclusion criteria included patients who were diagnosed cervical cancer with no other gynecologic oncology or infectious disease. The median age was 51 years old (range 39–56 years).

Cell lines and antibodies

The human cervical cell lines SiHa (HPV16$^+$) and C33A (HPV16$^-$) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 ng/ml) in a humidified incubator at 37°C with 5% CO_2. LPS and IKKβ inhibitor SC-514 were ordered from Sigma-Aldrich (USA). Anti-IKKβ and anti-IκBα antibodies were from Millipore (USA). Anti-p53 and anti-p21 antibodies were from Santa Cruz Biotechnology (USA). Anti-β-actin antibodies were from Auragene (USA).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tumor tissue slides were deparaffinized using xylene and graded ethyl alcohol and then rinsed in water. Antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer in a microwave oven for 10 min and cooling at room temperature. The slides were then incubated with 0.05% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, followed by sequential treatment in a humidified chamber after quenching endogenous peroxides with 3% H_2O_2 in MeOH: blocking serum with avidin for 20 min, first antibody overnight at 4°C, secondary antibody for 20 min, hydrogen peroxidase for 15 min, and peroxidase substrate solution for 20 min at room temperature. The stained slides were then counterstained with hematoxylin and cover slipped. Staining was assessed by two independent investigators in a blind manner to reach a consensus. Staining intensity was recorded as negative (0), weakly positive (+1), positive (+2), and strongly positive (+3).

Western blot analysis

Cells were harvested and washed twice with cold PBS, and then resuspended and lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 ng/ml phenylmethylsulfonyl fluoride, 0.03% aprotinin, 1 μM sodium orthovanadate) at 4°C for 30 min after treated with LPS or SC-514. Lysates were centrifuged for 10 min at 14,000 ×*g*, and supernatants were stored at −80°C as whole cell extracts. Total protein concentrations were determined with Bradford assay. Proteins were separated on 12% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin and incubated with the indicated primary antibodies. Corresponding horseradish peroxidase-conjugated secondary antibodies were used against each primary antibody. Proteins were detected using the chemiluminescent detection reagents and films.

Statistical analysis

All statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., Armonk, NY, USA). All results are expressed as mean ± standard deviation (SD). Statistical analysis was performed with one-way analysis of variance or least significant difference. Correlation analysis was analyzed with Chi-square test or, in the case of low expected frequencies, by the Fisher's exact test. A value of $P < 0.05$ was considered statistically significant.

Results

IKKβ is highly expressed in the tissues of human cervical squamous carcinoma

A total of 36 pairs of cervical squamous carcinoma and adjacent normal tissues were collected to detect their expression of IKKβ protein with immunohistochemical staining. The representative results are shown in Figure 1. As shown in Table 1, the positive rate of IKKβ protein in cervical squamous carcinoma (100%) was significantly higher than that in adjacent normal tissues (64.3%). In addition, the expression of IKKβ was no significant difference between tumor differentiation, tumor size, and invasive depth with IKKβ expression [Table 2].

Effect of LPS on the expressions of IKKβ, IκBα, p53 and p21 proteins in cervical cancer cells

To evaluate the expression of IKKβ, IκBα, p53, and p21 protein on cervical cancer cell line SiHa (HPV16$^+$) and C33A (HPV16$^-$) with LPS treatment at 2 μg/ml for 15, 35, and 60 min,[11] as shown in Figure 2a, the expression of IKKβ protein increased but IκBα, p53, and p21 proteins decreased after LPS treatment in SiHa cells. However, no significant difference on the expressions of IKKβ, IκBα, p21, and p53 proteins after LPS treatment in C33A cells [Figure 2b]. HPV16

Table 1: Summary of IKKβ protein expression in the cervical squamous carcinoma and adjacent normal tissue

Group	Total cases	Positive cases	Positive rate (%)	χ^2	*P*
Squamous carcinoma	36	36	100	20.571	0.000
Adjacent normal tissue	36	20	64.3		

may mediate the expressions of IKKβ, IκBα, p53, and p21 proteins by LPS treatment in cervical cancer cell line from our observation.

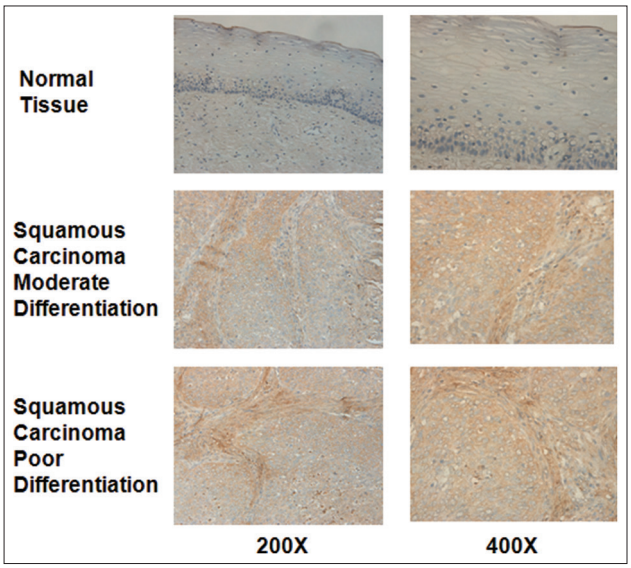

Figure 1: IKKβ is highly expressed in the tissues of human cervical squamous carcinoma. The expressions of IKKβ protein in the tissues of human cervical squamous carcinoma were examined by immuno-histochemistry staining. The representative expression of IKKβ protein in the cervical squamous carcinoma and adjacent normal tissue was shown.

IKKβ mediates the downregulation of p53 and p21 by lipopolysaccharide in human papillomavirus 16$^+$ cervical cancer cells

To assess the function of IKKβ on the downregulation p53 and p21 by LPS, SiHa and C33A cell lines were incubated with SC-514 (IKKβ inhibitor) before LPS treatment. As shown in Figure 3a, SC-514 totally reversed the upregulation of IKKβ and downregulation of p53 and p21 by LPS in SiHa cells. However, SC-514 had no effects on the expressions of p53 proteins in C33A cells before and after LPS treatment [Figure 3b]. These data showed that IKKβ may mediate the downregulation of p53 and p21 by LPS in HPV16-positive cervical cancer cells.

Discussion

In the present study, the data showed that the positive rate of IKKβ protein expression in cervical squamous carcinoma tissue was significantly higher than in adjacent normal tissues. The expression of IKKβ was no significant difference between tumor differentiation, size, and invasive depth of cervical cancer in our observation. Several cancer studies reported the increased expression of IKKβ relative to the nuclear localization and phosphorylation in the tissues and cell lines of head and neck squamous cell carcinomas (HNSCC). The IKKβ plays the important function with IKKα to activate NF-κB and EGFR/AP1

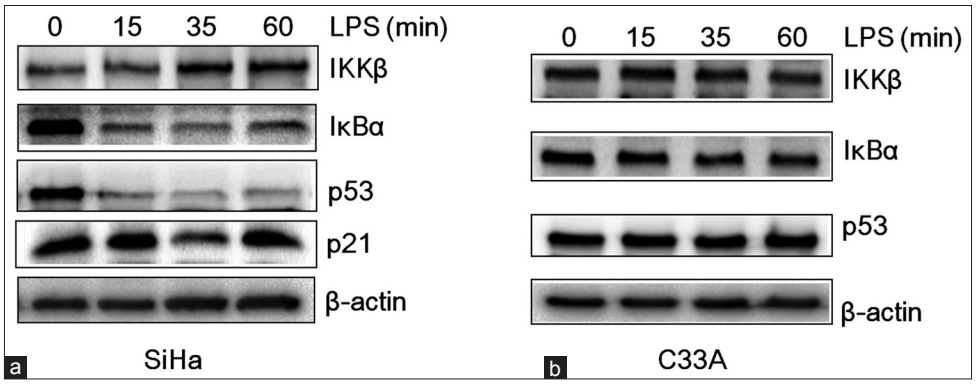

Figure 2: The effect of lipopolysaccharide on the expressions of IKKβ, IκBα p53 and p21 proteins in cervical cancer cells. Cells were treated with lipopolysaccharide at 2 μg/ml for 15, 35, and 60 min. Proteins were detected by Western blot with the indicated antibodies. The representative results were shown. (a) In SiHa cell line (HPV16$^+$); (b) In C33A cell line (HPV16$^-$).

Table 2: Correlation of IKKβ protein expression with differentiation, tumor size, and invasive depth

Group	Total cases	–	+	++	+++	Positive rate (%)	*P*
Differentiation							>0.05
Moderate	29	0	5	14	10	100	
Poor	7	0	0	2	5	100	
Tumor size (cm)							
<4	30	0	5	15	10	100	
≥4	6	0	0	1	5	100	
Invasive depth							
<1/2 myometrium	28	0	5	15	8	100	
≥1/2 myometrium	8	0	0	1	7	100	

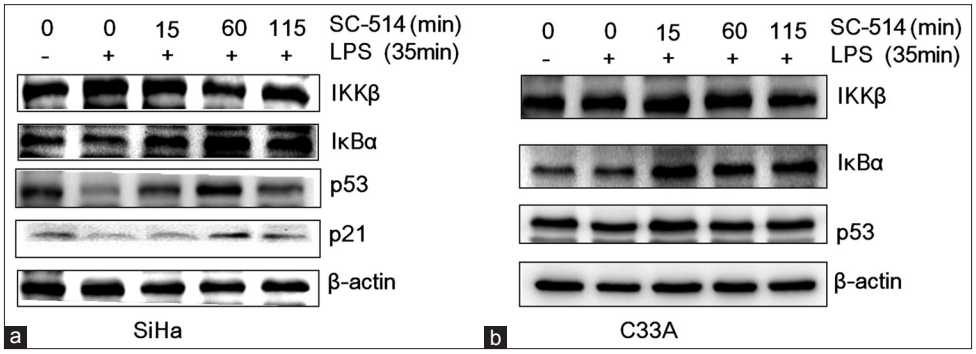

Figure 3: IKKβ mediates the downregulation of p53 and p21 by lipopolysaccharide treated in human papillomavirus 16$^+$ cervical cancer cells. Cells were incubated with SC-514 at 100 μM before lipopolysaccharide treatment. Proteins were detected by Western blot with the indicated antibodies. The representative results were shown. (a) In SiHa cell line (HPV16$^+$); (b) In C33A cell line (HPV16$^-$).

signaling and promote survival and migration of HNSCC.[12] IKKβ is also highly expressed in the tissues of human ovarian cancer and associated with poor survival rate, but no related to tumor stage, grade, and level of residual disease at the previous studies.[13] Many studies indicated that IKKβ is also highly expressed in the tissues of human liver cancer and the malignant properties of liver cancer effected by the level of IKKβ protein.[14] Therefore, IKKβ may be a potential biomarker for cancer therapy and diagnosis in future.

The results exhibited that LPS treatment increased the expression of IKKβ but decreased the expression of IκBα, p53, and p21 in SiHa but no different in C33A cell line. The level of IKKβ was reversed by the SC-514 treatment, upregulation inhibitor, in SiHa cell line (HPV16$^+$) but not in C33A cell line (HPV16$^-$). From previous reports, IKKβ targeted inhibition of IKKβ to block NF-κB pathway can suppress the growth of cancer cells or sensitize tumor cells to chemotherapy.[15] Theoretically, HPV play an important role for cervical carcinogenesis, and IKKβ could be as indicator for cervical cancer treatment or chemotherapy.

SC-514 is a new selective, reversible, and competitive with adenosine triphosphate (ATP) of IKKβ inhibitor and can attenuate RANKL-induced osteoclastogenesis and NF-κB activation.[16] Treatment of oral squamous cell carcinoma SCC25-PD cells with the SC-514 effectively inhibits RelA phosphorylation on Ser536, reverses nuclear-translocation of RelA, markedly blocks NF-κB gene activation and diminished cellular invasiveness.[17] Up-to-date, lots of IKKβ inhibitors have been developed including ATP analogs (PS-1145 and ML120B), allosteric inhibitors (BMS-345541), and thiol-reactive compounds (parthenolide and arsenite).[5,18-21] PS-1145 and ML120B show strong anticancer activities against chronic myelogenous leukemia, diffuse large B-cell lymphoma, multiple myeloma, and prostate cancer in the preclinical studies.[22-24] BMS-345541 exhibits an antitumor activity in a xenograft model of melanoma by inducing cell apoptosis[25] and sensitizes cancer cells to ionizing radiation or TRAIL.[26,27] Although it is a relatively safe agent that does not cause significant normal tissue damage *in vivo*,[28,29] it remains to be determined the antitumor effects and safety of IKKβ inhibitors in the clinical trial.

In conclusion, this study demonstrates that inhibition of IKKβ reverses the downregulation of p53 and p21 by LPS in HPV16$^+$ cervical cancer cells, suggesting that IKKβ may mediate the downregulation of p53 and p21 by LPS and be a new therapeutic target in HPV16$^+$ cervical cancer cells.

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Conflicts of interest

There are no conflicts of interest.

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