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

Zhi-Hui Tan, Yu Zhang, Yan Tian, Wei Tan, Ying-Hua Li
Department of Gynecology, Xiangya Hospital, Central South University, Changsha, Hunan 410078, China

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-mediated 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 plays an important role 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...
positive or negative cervical cancer cells through detecting the expression of IKKβ, IkBα, 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% CO₂. 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% H₂O₂ 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, IkBα, p53 and p21 proteins in cervical cancer cells**

To evaluate the expression of IKKβ, IkBα, 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, as shown in Figure 2a, the expression of IKKβ protein increased but IkBα, p53, and p21 proteins decreased after LPS treatment in SiHa cells. However, no significant difference on the expressions of IKKβ, IkBα, p21, and p53 proteins after LPS treatment in C33A cells [Figure 2b].

| Group                  | Total cases | Positive cases | Positive rate (%) | χ²   | 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.

**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 2µg/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    |             |    |    |    |     |                    |     |
| Moderate           | 29          | 0  | 5  | 14 | 10  | 100                | >0.05 |
| 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                |     |

**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.
Inactivation of the CYLD deubiquitinase by HPV E6 of IKK remains to be determined the antitumor effects and safety or TRAIL. apoptosis activity in a xenograft model of melanoma by inducing cell lymphoma, multiple myeloma, and prostate cancer in the PS‑1145 and ML120B show strong anticancer activities thiol‑reactive compounds (parthenolide and arsenite). and ML120B), allosteric inhibitors (BMS‑345541), and have been developed including ATP analogs (PS‑1145 cellular invasiveness. RelA, markedly blocks NF‑ phosphorylation on Ser536, reverses nuclear‑translocation of attenuation RANKL‑induced osteoclastogenesis and NF‑... mediated the downregulation of p53 and p21 by lipopolysaccharide treated in human papillomavirus 16+ cervical cancer cells. 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.

**Financial support and sponsorship**

This work was supported by funds from the Chinese National Natural Science Foundation No. 81101966.

**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Siegel R, Ma J, Zou Y, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9‑29. doi: 10.3322/caac.21208.
2. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003;348:518‑27. doi: 10.1056/NEJMoa021641.
3. Gaykalova DA, Manola JB, Ozawa H, Zizkova V, Morton K, Bishop JA, et al. NF‑κB and sta3 transcription factor signatures differentiate HPV‑positive and HPV‑negative head and neck squamous cell carcinoma. Int J Cancer 2015;137:1879‑89. doi: 10.1002/ijc.29558.
4. An J, Mo D, Liu H, Veena MS, Srivatsan ES, Massoumi R, et al. Inactivation of the CYLD deubiquitinease by HPV E6 mediates hypoxia‑induced NF‑κappaB activation. Cancer Cell 2008;14:394‑407. doi: 10.1016/j.ccr.2008.10.007.
5. Havid L, Delvenne P, Fraré P, Boniver J, Giannini SL. Differential production of cytokines and activation of NF‑κappaB in HPV‑transformed keratinocytes. Virology 2002;298:271‑85. doi: 10.1006/viro.2002.1468.
6. Cildir G, Low KC, Tergaonkar V. Noncanonical NF‑κB signaling in health and disease. Trends Mol Med 2016;22:414‑29. doi: 10.1016/j.molmed.2016.03.002.
7. Herrington FD, Carmody RJ, Goodyear CS. Modulation of NF‑κB signaling as a therapeutic target in autoimmunity. J Biomol Screen 2016;21:223‑42. doi: 10.1177/1087057116714756.
8. Bosman MC, Schuringa JJ, Vellenga E. Constitutive NF‑κB activation in AML: Causes and treatment strategies. Crit Rev Oncol Hematol 2016;98:35‑44. doi: 10.1016/j.critrevonc.2015.10.001.
9. Xia Y, Padre RC, De Mendoza TH, Bottero V, Tergaonkar VB, Verma IM. Phosphorylation of p53 by IkappaB kinase 2 promotes its degradation by beta‑TrCP. Proc Natl Acad Sci U S A 2009;106:2629‑34. doi: 10.1073/pnas.0812255106.
10. Yang PM, Huang WC, Lin YC, Huang WY, Wu HA, Chen WL, et al. Loss of IKKbeta activity increases p53 stability and p21 expression leading to cell cycle arrest and apoptosis. J Cell Mol Med 2010;14:687‑98. doi: 10.1111/j.1582‑4934.2009.00712.x.

**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+).
11. Kobori M, Yang Z, Gong D, Heissmeyer V, Zhu H, Jung YK, et al. Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex. Cell Death Differ 2004;11:123-30. doi: 10.1038/sj.cdd.4401325.

12. Nottingham LK, Yan CH, Yang X, Si H, Coupar J, Bian Y, et al. Aberrant IKKa and IKKβ cooperatively activate NF-κB and induce EGFR/AP1 signaling to promote survival and migration of head and neck cancer. Oncogene 2014;33:1135-47. doi: 10.1038/onc.2013.49.

13. Hernandez L, Hsu SC, Davidson B, Birrer MJ, Kohn EC, Annunziata CM. Activation of NF-κB signaling by inhibitor of NF-κB kinase increases aggressiveness of ovarian cancer. Cancer Res 2010;70:4005-14. doi: 10.1158/0008-5472.CAN-09-3912.

14. Jiang R, Xia Y, Li J, Deng L, Zhao L, Shi J, et al. High expression levels of IKKalpha and IKKbeta are necessary for the malignant properties of liver cancer. Int J Cancer 2010;126:1263-74. doi: 10.1002/ijc.24854.

15. Shao L, Wu L, Zhou D. Sensitization of tumor cells to cancer therapy by molecularly targeted inhibition of the inhibitor of nuclear factorκB kinase. Cancer Res 2010;126:1263-74. doi: 10.1002/ijc.24854.

16. Liu Q, Wu H, Chim SM, Zhou L, Zhao J, Feng H, et al. SC-514, a selective inhibitor of IKKβ, attenuates RANKL-induced osteoclastogenesis and NF-κB activation. Biochem Pharmacol 2013;86:1775-83. doi: 10.1016/j.bcp.2013.09.017.

17. Johnson J, Shi Z, Liu Y, Stack MS. Inhibitors of NF-kappaB reverse cellular invasion and target gene upregulation in an experimental model of aggressive oral squamous cell carcinoma. Oral Oncol 2014;50:468-77. doi: 10.1016/j.oraloncology.2014.02.004.

18. Castro AC, Dang LC, Soucy F, Grenier L, Mazdiyasni H, Hottelet M, et al. Novel IKK inhibitors: Beta-carbolines. Bioorg Med Chem Lett 2003;13:2419-22. doi: 10.1016/S0960-894X(03)00408-6.

19. Wen D, Nong Y, Morgan JG, Gangurde P, Bielecki A, Dasilva J, et al. A selective small molecule IkappaB Kinase beta inhibitor blocks nuclear factor kappaB-mediated inflammatory responses in human fibroblast-like synoviocytes, chondrocytes, and mast cells. J Pharmacol Exp Ther 2006;317:989-1001. doi: 10.1124/jpet.105.097584.

20. Kapahi P, Takahashi T, Natoli G, Adams SR, Chen Y, Tsien RY, et al. Inhibition of NF-kappa B activation by arsenite through reaction with a critical cysteine in the activation loop of Ikappa B kinase. J Biol Chem 2000;275:36062-6. doi: 10.1074/jbc.M007204200.

21. Kwok BH, Koh B, Ndubuisi MI, Elossoin M, Crews CM. The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IkappaB kinase. Chem Biol 2001;8:759-66. doi: 10.1016/S1074-5521(01)00049-7.

22. Cilloni D, Messa F, Arruga F, Defilippi I, Morotti A, Messi E, et al. The NF-kappaB pathway blockade by the IKK inhibitor PS1145 can overcome imatinib resistance. Leukemia 2006;20:61-7. doi: 10.1038/sj.leu.2403998.

23. Yemelyanov A, Gasparian A, Lindholm P, Dang L, Pierce JW, Kisseljov F, et al. Effects of IKK inhibitor PS1145 on NF-kappaB function, proliferation, apoptosis and invasion activity in prostate carcinoma cells. Oncogene 2006;25:387-98. doi: 10.1038/sj.onc.1209066.

24. Rajmani RS, Gandham RK, Gupta SK, Sahoo AP, Singh PK, Saxena S, et al. Administration of IkappaB-kinase inhibitor PS1145 enhances apoptosis in DMBA-induced tumor in male Wistar rats. Cell Biol Int 2015;39:1317-28. doi: 10.1002/cbin.10510.

25. Yang J, Amiri KI, Burke JR, Schmid JA, Richardson A. BMS-345541 targets inhibitor of kappaB kinase and induces apoptosis in melanoma: Involvement of nuclear factor kappaB and mitochondria pathways. Clin Cancer Res 2006;12(3 Pt 1):950-60. doi: 10.1186/1078-0432.CCR-05-1220.

26. Wu L, Shao L, An N, Wang J, Pazhanisamy S, Feng W, et al. IKKβ regulates the repair of DNA double-strand breaks induced by ionizing radiation in MCF-7 breast cancer cells. PLoS One 2011;6:e18447. doi: 10.1371/journal.pone.0018447.

27. Berger A, Quast SA, Plötz M, Kammermeier A, Eberle J. Sensitization of melanoma cells for TRAIL-induced apoptosis by BMS-345541 correlates with altered phosphorylation and activation of Bax. Cell Death Dis 2013;4:e477. doi: 10.1038/cddis.2012.198.

28. Gillooly KM, Pattoli MA, Taylor TL, Chen L, Cheng L, Gregor KR, et al. Periodic, partial inhibition of IkappaB Kinase beta-mediated signaling yields therapeutic benefit in preclinical models of rheumatoid arthritis. J Pharmacol Exp Ther 2009;331:349-60. doi: 10.1124/jpet.109.156018.

29. Fuchs O. Transcription factor NF-xB inhibitors as single therapeutic agents or in combination with classical chemotherapeutic agents for the treatment of hematologic malignancies. Curr Mol Pharmacol 2010;3:98-122. doi: 10.2174/1874467211003030098.