Original Article

Lipopolysaccharide (LPS) regulates peroxide to affect the development of nasopharyngeal carcinoma by suppressing Act1 expression

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Abstract: Background: Lipopolysaccharide (LPS) is located in the outer leaflet of Gram-negative bacteria which consists of three basic units: Lipid A, core polysaccharide, and O-specific side chain. Act1, an NF-κB activator, is initially proposed by Li et al. and is widely expressed in the thymus, heart, lung, kidney, blood, liver, colon, and placenta tissues. Peroxisome proliferator-activated receptors (PPARs) have been reported to be involved in the regulation of tumor proliferation and apoptosis, which is considered to be a new anticancer treatment. Our study aims to reveal the relationship among LPS, Act1, peroxides, and NPC. Methods: N,N'-Dinitrosopiperazine (DNP) was used to model disease in humans. Tumor volume, weight, and the survival rates were measured in wild type and Act1−/− C57BL/6 mice. In addition, we examined the effects of LPS on Act1 expression, tumor cell migration, and invasion in NPC 5-8F cells. We further knocked down Act1 by RNA interference (RNAi) and detected PPARγ expression, tumor cell migration, and invasion in 5-8F cells. Additionally, clinical samples were used to test the expression of Act1 in different stages of NPC, in turn analyzing the prognostic factors. Results: In NPC mice model induced by DNP, the survival rates of Act1−/− mice were higher than that of control C57 mice, and both the tumor number and weight were significantly decreased in Act1−/− mice. We further showed that LPS treatment significantly decreased Act1 expression, and suppressed tumor cell migration and invasion in 5-8F cells. Silencing of Act in NPC cells resulted in decreased PPARγ expression and increased tumor cell migration and invasion in vitro. Furthermore, this study showed that high Act1 expression in NPC tissues and correlated with the severity of NPC patients. Conclusion: Taken together, our findings suggest that LPS can reduce the expression of PPARγ by decreasing the expression of Act1, thereby suppressing tumor cell migration and invasion of 5-8F cells, which attenuates the pathological process of NPC.

Keywords: LPS, Act1, PPARγ, NPC

Introduction

The incidence of malignancy is increasing year by year, which become the main threats to human health. Currently, clinical treatment for malignancy includes three ways: surgical resection [1-3], radiotherapy [4-6], and chemotheraphy [7-9]. Given that each approach has advantages and disadvantages, the combination of various treatment modalities is the major option for tumor therapy [10]. Nasopharyngeal carcinoma (NPC) is a kind of cancer that derives from the epithelium of nasopharynx with high risk of metastasis and poor prognosis. NPC is rare in most parts of the world but occurs with a high incidence in Southern China, especially the Cantonese, accounting for 60% of NPC in China. Even migrate to Singapore, the United States, Canada and other countries, the incidence of NPC is higher than that of the local people. The prognosis of young patients is better than that of the older patient [11]. Lipopolysaccharide (LPS) is located in the outer leaflet of Gram-negative bacteria with at the thickness of 8-10 nm, which consists of three basic units: Lipid A, core polysaccharide, and O-specific side chain. NF-κB activator Act1, also termed TRAF3 interacting protein 2 (TRAF3IP2) or connection to IκB kinase and stress-activated protein kinases (CIKS), plays important roles in immune signaling pathways. Act1 is a cytoplasmic protein contains four domains comprised of 574 amino acids: a coiled-coil domain which contains the similar expression to fibroblast...
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growth factor genes/IL-17R (SEFIR) at the C-terminus, a helix-loop-helix domain at the N-terminus, and two tumor necrosis factor receptor-associated factor (TRAF) binding sites [12, 13]. Act1 is an important negative modulator of B cell-mediated humoral immune responses by regulating CD40-CD40L and BAFF-BAFFR signaling. The Act1-deficient mice exhibited increased Peripheral B cells, enlarged lymph nodes and spleens, hypergammaglobulinemia, and several autoantibodies. Studies have identified Act1 as a key component of IL-17 signaling pathway and are essential for IL-17-dependent autoimmune and infectious responses [14]. Li et al [15] have demonstrated that Act1 could activate IkB kinase, leading to the release of NF-kB from its complex with IkB, which contributes to NF-kB activation. In addition, Act1 also activates activating transcription factor (ATF) and activator protein 1 (AP-1) through Jun kinase (JNK). Studies have shown that Act1-deficient epithelial cells reduce IL-25-induced Th2 immune response and lung inflammation. Qian et al [16] have reported that Act1-deficient mice have much less inflammatory disease in dextran sodium sulfate (DSS)-induced colitis.

Recent studies reveal that Peroxisome proliferator-activated receptors (PPARs) are involved in the regulation of tumor proliferation and apoptosis, which considered to be a new anticancer treatment [17]. PPAR receptors include PPARα, PPARβ, and PPARγ. PPARγ is a ligand-activated transcription factor that plays important roles in regulating hormone balance and inhibiting the inflammatory process. Oxidized fat from oxidized low density lipoprotein can effectively activate PPAR and thus change the expression of inflammatory factors. Besides its metabolic and anti-inflammatory properties, growing evidences indicate that PPARγ impacts tumor cell proliferation and differentiation [18, 19], and its ligand significantly inhibit the growth of NPC cells and the expression of carcinoembryonic antigen. However, whether there is a link between Act1, PPARγ, and NPC upon inflammatory factors stimulation such as LPS remains unclear.

Materials and methods

Patients

Tissue specimens from patients with NPC were collected from July 2014 to December 2016 in our hospital. According to the 2009 edition of the International Anti-Cancer Association (UICC) TNM standards, patients were divided into different clinical stages. All data were obtained with the written informed consent of the participants prior to their inclusion in the study, according to the principles of the Declaration of Helsinki and approved by the Hospital Ethics Commission in the Second Affiliated Hospital of Guangxi Medical University.

Mice and cell culture

A total of 50 adult C57BL/6 mice (25 male mice, 25 female mice, 8-week old) were purchased from the Laboratory Animal Center of Sun Yat-sen University used as controls. Act1-deficient mice in the C57BL/6J background were purchased from Jackson laboratory. 5-8F cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China).

NPC mice model

8-week-old mice, weight 18-20 g, received subcutaneous injections of 0.5% N,N’-Dinitroso-piperazine (DNP, 15 mg/kg body weight) in water twice per week for 6 consecutive months. NPC were occurred after 6 months, the rate of NPC formation can reach at 85% with the prolongation of the survival time of animals. During the different stages of carcinogenesis, the atypical hyperplasia of basal cells, squamous epithelial cells, and columnar epithelial cells was highly correlated with carcinogenesis.

Immunohistochemistry

Tumor tissues were collected from mice and fixed in 10% neutral formalin overnight. Following dehydration, the tumor samples were embedded in paraffin and cut into sections of 4 μm. After baked for 1 h at 65°C, the sections were routinely deparaffinized and then treated with 0.3% hydrogen peroxide for 30-60 min to block endogenous peroxidases. After three washes in PBS, the sections were repaired for 5 min in 0.01 M citrate buffer (pH 6.0) in a microwave. Then the sections were blocked with 10% fetal bovine serum for 30 min at room temperature and incubated at 4°C overnight with anti-Ki67 (1:200, Cell Signaling Technology, USA). The following day, the sections were washed with PBS and then a secondary antibody (1: 200) was added and incubated for 1 h at 37°C. Finally, the images were visualized with stereomicroscope following DAB staining and hematoxylin staining.
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For Western blot, tissues were lysed in lysis buffer (Beyotime Biotechnology, China) and BCA assay (Thermo Fisher Scientific, USA) was used for protein quantitation according to the manufacturer’s instructions. The equivalent proteins were mixed with 5× sample loading buffer and loaded on SDS-PAGE gels that were run at 80 V for 20 min, then at 120 V for about 1 h until the bromophenol blue runs out of the bottom of the gel. The gel was removed and

Figure 1. Act1 deficient inhibits the development of NPC. A. Volumetric change in tumor sizes of the C57BL/6 and Act1−/− mice induced by DNP. B. Statistical analysis of tumor weight in control and Act1−/− mice. C. The survival rates of C57BL/6 and Act1−/− mice induced by DNP. D. Ki67 immunostaining in control and Act1−/− mice. Data represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. LPS delays the development of NPC by inhibiting Act1 expression. A. LPS treatment reduced the expression of Act1. B. LPS treatment attenuated tumor cell migration. C. LPS treatment inhibited tumor cell invasion.
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transferred to the polyvinylidene fluoride (PVDF) membrane. Then the PVDF membranes were blocked with 5% non-fat milk for 1 h at room temperature, blotted with indicated primary antibodies at 4°C overnight and the membrane was rinsed with TBST three times with each time for 5 min. The membrane and secondary antibody conjugated with HRP were incubated with gently agitation at room temperature for 1 h and was subsequently washed with TBST for three times (5 min each time). The membrane proteins were attached to the X-ray film for quick exposure in a darkroom and developed.

Cell scratch test

A scratch test was performed to evaluate the mobility of tumor cells. In brief, 3×10^5-8×10^5 cells/well were seeded in a 6-well plate and cultured overnight. Straight scratches were then made vertical to the line drawn on the back of the plate, and observed at every 12 h.

Transwell assay

Transwell assays were performed to assess the migration and invasion of tumor cells. Briefly, cells with indicated treatments were added into the upper compartment and cultured at 37°C in 5% CO₂. Complete DMEM culture medium with 5% FBS was added into the lower compartment. After 24 h culture, cells were washed, fixed with 4% paraformaldehyde and stained with 1% crystal violet. The number of cells that penetrated through the membrane was observed by inverted optical microscope and counted in five fields, and the average number of the field was calculated.

Statistical analysis

Statistical analyses were performed using SPSS 10.0 and Sigmaplot software. For immunohistochemistry staining, the gray value was analyzed by Image Pro Plus software. Statistical significance between different groups was assessed by ANOVA *P<0.05, **P<0.01, ***P<0.001.

Results

Deletion of Act1 inhibits the development of NPC

In order to further investigate the relationship between Act1 and NPC, DNP was used to model disease and tumor induction was detected in control C57BL/6 and Act1^-/- mice. As the results shown in Figure 1, NPC mice model induced by DNP, Act1 deficient mice showed decreased tumor volume and weight than that of control mice. In addition, the survival rates of Act1^-/- mice were much higher than that of control mice. Tumor proliferation assay showed that
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the expression of Ki67 in Act1⁻/⁻ mice was significantly lower than that of control group, indicating that Act1 deficient inhibits the development of NPC.

LPS attenuates the development of NPC by suppressing Act1 expression

Next, 1 mg/mL LPS was used to induce inflammation in 5-8F cells and DMSO in the same dose was used as control. We found that LPS treatment significantly decreased Act1 expression (Figure 2A) and suppressed tumor cell migration (Figure 2B) and invasion (Figure 2C) in 5-8F cells.

Knockdown of Act1 decreases PPARγ expression in 5-8F cells

Our above data revealed that decreased Act1 expression significantly changed the mobility of tumor cells, which affects the development of

Figure 4. Clinical significance of the expression of Act1 and PPARγ in NPC patients. A. Immunohistochemistry for Act1 and PPARγ expression in NPC patients. B. Effects of Act1 on over survival and distant-metastasis-free survival. C. Effects of Act1 on over survival and distant-metastasis-free survival.
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NPC cells. In addition, PPARγ has been reported to regulate tumor cell proliferation and differentiation. Therefore, it prompted us to consider whether there is a link between Act1 and PPARγ. To test this, we knocked down of Act1 by RNA interference (RNAi) and detected PPARγ expression in 5-8F cells. We found that Silencing of Act in NPC cells resulted in decreased PPARγ expression (Figure 3A) and increased tumor cell migration (Figure 3B) and invasion (Figure 3C) in 5-8F cells.

Clinical significance of the expression of Act1 and PPAR in NPC patients

Our data indicate that LPS decreases the expression of PPARγ by suppressing Act1 expression, thereby inhibiting tumor cell migration and invasion of 5-8F cells, which attenuates the pathological process of NPC. But the expression of Act1 and PPARγ had not been studied in clinic. Therefore, clinical samples were used to test the expression of Act1 in different stages of NPC, in turn analyzing the prognostic factors. In Figure 4A, we found Act1 and PPARγ expression in NPC tissues and correlated with the severity of NPC patients. As shown in Figure 4B, the overall survival and distant metastasis showed that the low expression of Act1 can effectively improve the survival and distal metastasis of patients, as shown in Figure 4C; the same method to detect PPAR can also effectively improve the survival rate of patients And remote transfer.

Discussion

NPC is a highly malignant tumor with complex pathogenesis. Abnormal expression of genes has been related to the progression of cancer. The occurrence and development of NPC is a typical multiple process which involves aberrant cell proliferation, apoptosis, invasion and metastasis. Act1 is thought to associate with many tumor diseases, and specific deletion of Act1 in astrocytes can ameliorate experimental autoimmune encephalomyelitis. Prakashsrinivas et al [20] have shown that Act1 deficient significantly inhibits the expression of IL-25-induced Th2-type cytokines (IL-4, IL-5, IL-13) and chemokines in a mouse model of allergic asthma. Ablation of Act1 in epithelial cells reduces IL-25-induced Th2 type immune responses and lung inflammation. The Act1-deficient mice exhibited increased Peripheral B cells, enlarged lymph nodes and spleens, hypergammaglobulinemia and autoantibodies. However, the effects of Act1 on NPC have not been reported. Here, our findings revealed that Act1-deficient mice attenuates the development of NPC. LPS, as the inflammation-inducing agent, often leads to inflammation and aggravates the occurrence of disease in the process of tumorigenesis. We found that LPS treatment significantly decreased Act1 expression and suppressed tumor cell migration and invasion. Act1 has been identified as an important downstream adaptor protein of IL-signaling, which contributes to the activation of NF-κB and MAPKs signaling pathway. Anthony et al [20] have found that knockout of Act1 can attenuate AngII-induced myocardial hypertrophy and cardiac fibrosis, suggesting that Act1 may also have an impact on hypertension. Naveen [21] et al have confirmed that Act1 is involved in the regulation of aldosterone-induced myocardial cell growth and fibroblasts proliferation and migration. Based on these, we speculated that Act1 may affect downstream cell growth regulators.

PPARγ is a ligand-dependent transcription factor that regulates diverse aspects of development and homeostasis including the occurrence of tumor and maintenance of glucose in vivo [22]. Related research showed that PPARγ plays an important role in antitumor process by promoting cell apoptosis, thereby increasing the P27kip1 expression in gastric cancer in a P53-dependent manner. In addition, PPARγ activator troglitazone effectively inhibits the growth of colon cancer cells [23, 24]. In our study, we found significantly decreased PPARγ expression with the decrease of Act1 in NPC cells. We further observed that depletion of Act1 results in increased tumor migration and invasion in 5-8F cells. It is concluded that the distant metastasis of NPC cells are also weakened. Based on this, clinical samples were used to detect the expression of Act1 and PPARγ and the in vivo data were consistent with the in vitro data.

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Disclosure of conflict of interest

None.
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