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Original Article

EBV-LMP1 regulating AKT/mTOR signaling pathway and WWOX in nasopharyngeal carcinoma

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Abstract: Our previous studies found the expression of tumor suppressor gene WWOX was reduced in nasopharyngeal carcinoma (NPC), and WWOX expression gradually declined with the progress and lymph node metastasis in patients. These suggested that WWOX was related with the development of NPC. AKT/mTOR signaling pathway was considered the primary pathway of cancer cell survival. AKT/mTOR pathway and WWOX had been found to be closely related. NPC was closely related to infection of Epstein-Barr virus (EBV). The study mainly used oncogene LMP1 of EBV as a starting point to explore whether LMP1 regulated the AKT/mTOR signaling pathway and WWOX gene. Western blot and qPCR were used to detect the expression of AKT/mTOR pathway (AKT, p-AKT, p70S6K and p-p70S6K) and WWOX in nasopharyngeal carcinoma cell lines CNE1 and CNE1-LMP1, and accessed relationship of LMP1 with AKT/mTOR and WWOX. Research of correlation between LMP1 and WWOX gene expression suggested that in CNE1-LMP1 cells, WWOX gene and protein levels were decreased compared with CNE1 cells (P=0.025, P=0.042, respectively). The difference was statistically significant, and suggested that LMP1 expression correlated with WWOX. Research of correlation between LMP1 and AKT/mTOR signaling pathway demonstrated that when cell line CNE1-LMP1 was compared with CNE1 in AKT/mTOR pathway key protein of AKT, p-AKT, p70S6K and p-p70S6K expression, P values were 0.075, 0.008, 0.124, 0.034, respectively, and expression of p-AKT, p-p70S6K in CNE1-LMP1 were higher than CNE1, which were significantly different from each other. It suggested AKT/mTOR pathway was regulated by LMP1. WWOX gene and AKT/mTOR signaling pathway were regulated by the EBV-LMP1 oncogene.

Keywords: WWOX, LMP1, nasopharyngeal carcinoma, AKT/mTOR, signaling pathway

Introduction

High incidence of NPC is in southern China, the morbidity and mortality rates rank the highest in the world. Currently, the main treatment for NPC is radiotherapy, but the treatment method can lead to a series of complications, and the effect is not ideal. According to epidemiological statistics, nasopharyngeal cancer mortality was 6.61/100,000 in some provinces, such as Guangxi Zhuang Autonomous Region. The mortality rate for men and women were 9.53/100 000 and 3.40/10 million, namely that male and female mortality ratio was 2.80:1. NPC accounts for 5.60% of all cancer deaths, ranked No. 4 cancer deaths. It seriously impacts on people's living standards and quality of life. Genetic, epigenetic and environmental factors NPC plays an important role in the pathogenesis of EBV [1-4]. NPC is the typical genetic-environment-virus interaction of cancer, and the EB virus latent infection is considered to be one of the important environmental factors in the development of NPC. At present, due to the fact that the pathogenesis of NPC is not yet fully understood; there are many basic research concerned the development of relations EBV and NPC. Therefore, EBV is as a starting point to better understand the etiology and pathogenesis of NPC, screen early diagnosis biomarkers, and look for new targets for drug treatment. It is critical to improve the cure rate and survival rate of patients with NPC. Meanwhile, with the advances in technology in recent years and research on tumor suppressor genes (or oncogene) and in-depth study of signaling pathways, these factors create the conditions for people to understand the cancer etiology and pathogenesis.

Activation of oncogenes, inactivation of tumor suppressor genes and other factors are related to the development of NPC. The present gene and protein levels have become a hot research direction to seek for its mechanisms. EBV is an oncogenic virus. Studies have shown that the load and the detection rate of EBV-DNA and EBV-VCA-IgA were significantly higher in the serum of patients with NPC as compared to healthy people [4], also confirmed the development of EBV and NPC was closely related. EBV expression of genes included LMP1 (latent membrane protein 1), LMP2, EBNA1, EBNA2, etc., and LMP1 was currently the only proven oncogene playing an important role in the malignant transformation of cells.

AKT/mTOR signaling pathway was the very important pathway inside the cell in the tumor process, and played an extremely considerable biological functions including proliferation, growth, survival, angiogenesis, apoptosis, autophagy and so on [5-7]. The pathway abnormalities could cause a range of diseases, including cancer [8-10], neuropathy [11], autoimmune disease [12] and hematopoietic disorders [13].

In 2000, Bednarek et al. [14] cloned a new tumor suppressor gene WWOX on chromosome 16. The region located on chromosome 16q23.3-24.1. It was across the common chromosomal fragile sites FRA16D, which was easy to break or lose, and leaded inactivation of the adjacent gene in the region. WWOX encoded protein could induce apoptosis, and the genetic defects and many types of cancer were closely related. It was very similar to the fragile histidine triad (FHIT) gene, and thus was considered to be another new tumor suppressor gene after FHIT tumor suppressor gene.

Studies have shown that in NPC, EBV might play a role in tumor promotion [15] by AKT/mTOR pathway, and Yamamoto-Sugitani et al. [16] found that AKT/mTOR pathway could regulate the expression of WWOX in primary cutaneous T cell tumor. Therefore, based on our previous work, we speculated that WWOX and AKT/mTOR pathway might play an important role in the development of NPC, and EBV might regulate the expression WWOX by AKT/mTOR pathway.

Based on our previous work, we intended to employ EBV as a starting point. By qPCR and WB, CNE1 was as a reference object, which was compared with CNE1-LMP1 through the key genes (p-AKT, p-P70S6K) in the AKT/mTOR pathway and WWOX gene. It would help to provide new research ideas of revealing the exact mechanism of NPC promoted by EBV.

Materials and methods

Cell culture

CNE1 and CNE1-LMP1 cell lines were provided by Xiangya School of Medicine, Central South University, Hunan. Cells were cultured in growth medium (RPMI-1640 medium, 10% fetal bovine serum, 100 kU/l penicillin, 100 mg/l streptomycin) at 37°C, 5% CO_2 and a humidified atmosphere.

RT-PCR and western bolt

Total RNA was isolated by TRIzol reagent (Invitrogen, USA) according to the protocol supplied by the manufacturer, and quantified by spectrophotometry. Then, 1 μg of total RNA was reverse transcribed by Reverse transcription kits of the TIANGEN Biotech (Beijing) Company Limited according to its instructions. The sequences of primers were as follows: β-actin: TTGCCGACAGGATGCAGAAGGA (sense), and AGGTGGACAGCGAGGCCAGGAT (anti-sense). WWOX: TCGCAGCTGGTGGGTGTAC (sense), and AGCTCCCTGTTGCATGGACTT (anti-sense). GAPDH: GCACCGTCAAGGCTGAGAAC (sense), and TGGTGAAGACGCCAGTGGA (anti-sense). The β-actin gene and GAPDH gene were selected as the internal reference genes. Expected PCR product will be 129 base pairs for β-actin gene, 73 base pairs for WWOX gene, and 138 base pairs for GAPDH gene. Quantitative RT-PCR (WWOX and GAPDH) was performed with a SYBR Green PCR kit (Takara) using the StepOnePlus™ Real-Time PCR System (Life Technoligies, USA).

Cells were harvested and lysed in ice-cold lysis buffer. After centrifugation, the protein concentrations of supernatant were determined by BCA protein assay kit (Beyotime, China). Equal amounts of proteins were separated by SDS PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with Tris buffered saline which contained 0.1% Tween-20 and 5% skim milk. Then the membranes were incubated with antibod-

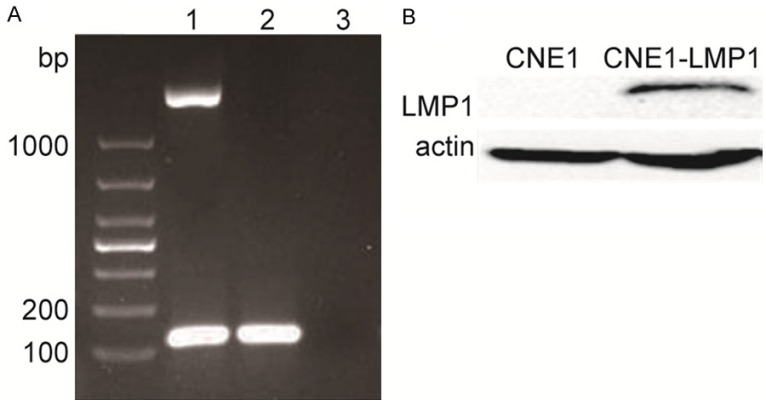

Figure 1. mRNA expression of LMP1 gene (A) (1 for CNE-LMP1, 2 for CNE1, 3 for H_2O. Product was 1300 bp. Internal reference was β-actin) and protein expression of LMP1 gene in cell (B).

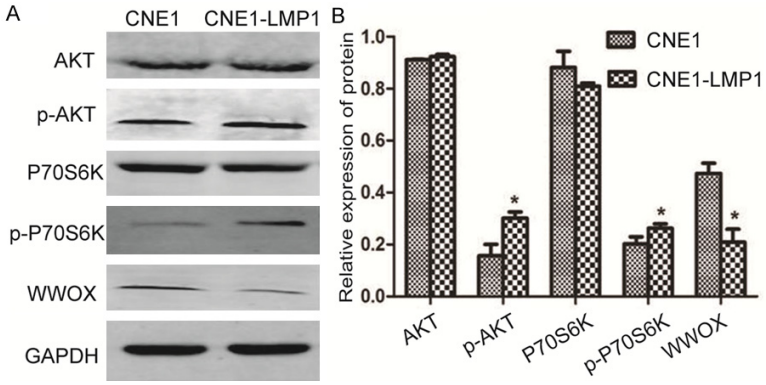

Figure 2. Relationship between LMP1 and AKT/mTOR pathway or WWOX (A and B).

ies at 4°C to avoid light overnight. The primary antibodies AKT (p-AKT), GAPDH, and secondary antibody were purchased from Cell Signaling Technology (USA), while P70S6K (p-P70S6K) were from Signalway Antibody LLC (USA), WWOX from ImmunoWay Biotechnology Company (USA). The next day, the PVDF membranes were placed in the fluorescent secondary antibody. Finally, sweeping instrument (LI-COR, USA) was used to detect specific proteins separated by electrophoresis.

LMP1 expression was first confirmed to be present in CNE1-LMP1 cells by WB and qPCR, and then the next step of the experiment was carried out. By qPCR and WB, CNE1 and CNE1-LMP1 cells were used to detect the key protein AKT (p-AKT), P70S6K (p-P70S6K) expression level and WWOX gene mRNA and protein expression level, in order to find that the relationship between LMP1 and AKT/mTOR pathway and WWOX.

Statistical analysis

The software SPSS16.0 was applied to analyze all the statistical analyses. Data were presented as mean ± SD. The T test was also used for comparison of two groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

LMP1 expression was confirmed to be present in CNE1-LMP1 cells by qPCR and WB (**Figure 1A** and **1B**). Next, AKT (p-AKT), P70S6K (p-P70S6K) protein and WWOX gene expression were detected in CNE1 and CNE1-LMP1 cells. In CNE1-LMP1 cells, the expression level of WWOX gene mRNA and protein was decreased when compared with CNE1 cells. *P* was 0.025 and 0.042, respectively, and there was statistical significance. It suggested that LMP1 was related to expression of WWOX gene (**Figure 2A**).

In CNE1 and CNE1-LMP1 cell line, the *P* value of AKT/mTOR pathway key protein AKT (p-AKT), P70S6K (p-P70S6K) expression level was 0.075, 0.008, 0.124, 0.034, respectively. AKT and P70S6K protein expression level were not found statistically significant difference. But the expression level of p-AKT and p-P70S6K protein in CNE1-LMP1 was higher than that in CNE1, and the difference was statistically significant, as shown in **Figure 2B**. It suggested that AKT/mTOR signaling pathway was regulated by LMP1.

Discussion

Tumor development involves complex multistep process, including activation of oncogenes and inactivation of tumor suppressor genes.

Research on tumor development mechanism at the gene and protein level, has become a hot research direction. NPC is more common in southern China, and is a local carcinoma. It belongs to epithelial malignancies. The top of the nasopharynx is the most common occurrence site, followed by the outer wall and pharyngeal recess. NPC presents with early cervical lymph node metastasis and distant metastasis. Because of its occult occurrence site, the majority of patients have been diagnosed in the advanced stage and the treatment results are poor. Thus, exploring the NPC from the level of gene expression on development mechanism has become an important part of NPC basic research. Gene expression studies include two aspects: transcriptomics and proteomics. They study gene mRNA expression and protein expression, respectively. Simultaneous detection of mRNA and proteins in cancer research has become a trend today.

WWOX gene was discovered in 2000, and was similar to FHIT tumor suppressor gene. Therefore, it was considered to be a new candidate tumor suppressor gene. A large number of experiments showed, WWOX protein involved in the regulation of apoptosis, proliferation and/or maturation, and the downregulation or absence of WWOX expression was closely related to epithelial cancer [17-25]. Restoring or increasing the expression of the gene could inhibit the development of tumor cells [17]. Study by Maeda et al. [26] was found that WWOX mRNA and protein expression of cell line were reduced or absent, and tissue samples found WWOX and depth of invasion, lymph node metastasis, and the various stages of clinical pathology were related. Our previous study also found, there were correlation in WWOX and BCL-2, P73, P53, and other apoptosis and signal transduction related gene [27-29]. These findings suggested that, WWOX expression might play a significant inhibition in tumor. Currently, domestic and international coverage was still less research about WWOX tumor suppressor gene in NPC. Its mechanism was not yet fully clear.

EBV was one of DNA oncogenic viruses, which encoded two important latent membrane proteins (LMP1, 2) and one nucleoprotein (EBNA1). Only LMP1 was confirmed as a tumor gene, which played a very important role in the tumorigenic process in NPC. LMP1 could cause NPC by survivin, NF-κB, AKT/mTOR, p38 MAPK, ERK1/2, JNK1/2 and other signal transduction pathways [15, 30-32]. Its carcinogenicity increased the risk of tumorigenesis, and its associated signaling pathways and biological function had become a hot research in molecular biology.

AKT/mTOR signaling pathway was intracellular transduction pathway, which played an extremely important biological functions in tumor cell proliferation, growth, survival, angiogenesis, apoptosis, autophagy and other processes [5-7]. The pathway was considered as the primary pathway of cancer cells to survive. The abnormalities of the pathway could cause a range of cancer. EBV was closely related to AKT/mTOR and might play a role in tumor promotion through the pathway [15, 33]. In addition, a study found that AKT/mTOR pathway could regulate WWOX expression of primary cutaneous T-cell tumors [16]. WWOX gene possibly played a tumor suppressor role through the interactions of apoptosis factor or signaling pathways [27, 34]. Therefore, we speculate LMP1 might regulate WWOX expression via AKT/mTOR pathway. Next, we would further study the content.

Research of correlation between LMP1 and AKT/mTOR signaling pathway showed when CNE1-LMP1 was compared with CNE1 by the key protein of AKT, p-AKT, p70S6K and p-p70S6K, *P* values were 0.075, 0.008, 0.124, 0.034, respectively, and expression of p-AKT, p-p70S6K in CNE1-LMP1 were higher than CNE1, which were significantly different from each other. It suggested AKT/mTOR pathway was regulated by LMP1. This result was similar to findings Chen et al. [15]. Namely, LMP1 activated AKT/mTOR signaling pathway changes, altered expression of protein phosphorylation, and activated p-AKT, p-P70S6K and other proteins to promote the growth and spread of cancer cells. LMP1, as the upstream oncogene of AKT/mTOR pathway, had an important regulatory role in the expression of the downstream pathway-related genes, and even might cause related diseases.

Research of correlation between LMP1 and WWOX gene, protein expression: in CNE1-LMP1 cells, WWOX gene and protein levels were decreased compared with CNE1 cells (*P*=0.025, *P*=0.042, respectively), which was significantly

different from each other, and suggested that LMP1 expression correlated with WWOX. Many studies had shown that, WWOX gene was as a tumor suppressor gene, and its reduced expression could promote the development of cancer. Our results suggested that virus oncogene might reduced expression of tumor suppressor genes, and thus contributed to the occurrence of cancer.

LMP1 upregulated phosphorylation levels of AKT/mTOR pathway and downregulated expression levels of WWOX, and then promoted the development of NPC. However, some important message pathways, which affected by the LMP1 and resulted in cell proliferation, aging, death and malignant transformation, needed to be further study. Next, we also need to further study whether LMP1 regulates WWOX through the AKT/mTOR pathway.

In summary, LMP1 gene might have multiple effects. It could reduce the expression of tumor suppressor gene, such as WWOX gene. At the same time, it could activate AKT/mTOR pathway to prompt the development of NPC. The results of the study provided an experimental basis for the further study of the molecular mechanism of LMP1.

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Disclosure of conflict of interest

None.

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