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FULL PAPER

Nonstructural Protein NP1 of Human Bocavirus 1 suppresses the growth of A549 cell by promoting autophagy

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SUMMARY

Human bocavirus 1 (HBoV1) refers to a human parvovirus causing acute respiratory tract infection in children. Bocaviruses encode an NP1 protein, which has 47% amino acid homology with NP1 of Minute Virus of Canines (MVC) and Bovine Parvovirus (BPV), but not with any protein of other parvoviruses. NP1 was found to induce apoptosis in Hela cells, which does not depend on viral replication and other protein expression. However, whether NP1 induces pulmonary cell death is unclear. In the present study, we investigate the impacts of NP1 on the autophagy and viability of A549 cells by expressing NP1.

The plasmid containing NP1 gene was transfected into A549 cells. The apoptosis of A549 was evaluated by apoptosis detection kit and expression of caspase3. Cell viability and cell migration were detected by CCK8 kit and cell scratch test, respectively. The autophagy-related proteins and HMGB1 were detected by Western blot after NP1 expression in transfected cells. The real-time PCR was employed to detect HMGB1 mRNA. The secretory HMGB1 in supernatant of cell culture was measured by ELISA kit.

The transient expression of NP1 did not induce apoptosis in A549 cells, but inhibited cell viability and migration. The expression of Beclin1 and LC3 II increased significantly and that of autophagy substrate P62 decreased dramatically upon transfection of NP1. The expression of NP1 reduced both levels of mRNA and protein HMGB1. The NP1 induced A549 autophagy was activated by STAT3 signaling pathway.

HBoV1 NP1 induced autophagy in A549 cells by activating phosphorylation of STAT3 signaling pathway and inhibited A549 cell viability. This study provides insight into further elucidating the replication mechanism of HBoV1.

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INTRODUCTION

Human bocavirus 1 (HBoV1) is an autonomously replicating human parvovirus that belongs to the genus *Bocaparvovirus* in the family *Parvoviridae* (Cotmore *et al.*, 2014). The genome of HBoV1 falls into two major functional regions, i.e., nonstructural and structural genes, encoding non-structural proteins NS1-4, non-structural protein 1 (NP1) and capsid protein VP1/VP2/VP3, respectively. The NP1 protein of HBoV is a highly conserved non-structural protein localized in the cell nucleus and plays a key role in viral DNA replication (Dong *et al.*, 2018).

The NP1 induces Hela cell death which is independent of viral replication and other protein expression (Sun *et al.*, 2013). Recently, the HBoV1 has been found to kill airway epithelial cells by activating genes that inhibit apoptosis and promote pyrolysis. This strategy seems to facilitate replication of HBoV1 and allow HBoV1 to establish persistent infections in human airway epithelial cells (Deng *et al.*, 2017b).

Autophagy, one of the two major metabolic pathways of cell catabolism, maintains homeostasis through fine regulation. In fact, its disorders have been found to be the basis of many diseases, including cancer (Croce and Yamamoto, 2019). However, autophagy plays a multifaceted role in the occurrence and progression of cancer. It can inhibit tumors in the early stage of tumorigenesis. For established cancers, autophagy can promote their survival and progress (Luo *et al.*, 2019). Under the control of cellular pathways (e.g., STAT3, MAPKs and PI3k/AKT/mTOR), each step of autophagy is performed by several autophagy-related proteins (ATG). Autophagy may be used by viruses to serve viruses (Wu *et al.*, 2017).

In this study, we demonstrated that NP1 suppressed the growth of A549 cells and increased the levels of autophagy-related proteins (e.g., LC3-II and beclin-1). NP1 induced autophagy by activating phosphorylation of STAT3. Furthermore, the levels of both P62 and High Mobility Group Box 1 (HMGB1) were down-regulated in A549 cells transfected with NP1. These findings indicated that NP1 may be involved in lung damage by up-regulating autophagy.

Key words:
HBoV1, NP1, autophagy, LC3, HMGB1.

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MATERIALS AND METHODS

Cell Lines and transfection procedure

Human non-small cell lung cancer cells A549 were purchased from Institute of Biochemistry and Cell Biology and cultured in F12K medium containing 10% fetal bovine serum (FBS). The cells were seeded in appropriate culture disks to reach 80~90% confluency at transfection. The NP1 gene was amplified from HBoV1 genome (GenBank: GU139423.1; preserved in our laboratory) and linked to eukaryotic expression vector pCMV-tag2B by restriction site *EcoR* I and *Hind* III. Cell transfection was performed using Lipofecta-mine 2000 (Invitrogen).

Antibodies

Anti-NP1 rabbit polyclonal serum was prepared by our laboratory. Anti-HMG1, anti-GAPDH and anti-caspase 3 rabbit polyclonal antibodies were purchased from Immunoway. Beclin 1, p62 and LC3 rabbit polyclonal antibody were provided by Proteintech. p-STAT3 and p-PRKA rabbit polyclonal antibody was purchased from Sangon Biotech.

Cell viability assay

Cell viability was detected by a Cell Counting Kit-8 (CCK-8, Yesen, China). In this study, A549 cells were suspended in F12K medium containing 10% of FBS and 3×10^3 cells/100 μL were seeded in 96-well plates. At the indicated time points, the cells were stained with CCK-8 (10 μL/well), and then incubated at 37°C for 90 min. Absorbance was analyzed by a microplate reader.

Cell migration assay

The A549 cells were inoculated into 6-well plate with 5×10^5/well. On the second day, the cells were scratched with a tip (about 0.5 cm wide) and washed with PBS. The cells were cultured in serum-free medium. Photographs were taken at 6, 8 and 12 hours after culture, and the migration distance of cells was measured.

Tunel apoptosis detection (FITC)

The apoptosis assay was performed using Tunel apoptosis detection (FITC) Kit (Yesen, China) following the manufacturer's protocol. The cells were analyzed with fluorescence microscope using FITC label. In the high FITC signals, the late apoptotic population was recognized as apoptotic cells.

Western Blot Analysis

RIPA lysis buffer was used to lyse the cell and then extract the protein. The same amounts of proteins were separated by 10% SDS-PAGE. Subsequently, the proteins were transferred to polyvinylidene diluoride membranes by a western blot system. The membranes were sealed with 5% skim milk and incubated with the primary anti-HMGB1, LC3, NP1, beclin1, P62, caspase 3, p-STAT3 and p-PRKA antibodies respectively. GAPDH was used as an internal control.

Real-time PCR assay for HMGB1 detection

Total RNA of different cell samples was extracted with Trizol. AMV reverse transcriptase (TAKARA) was then used to reverse transcription of RNA. Each cDNA sample was made in triplicates. In terms of real-time PCR amplifications, the Applied Biosystems 7500 Real-Time PCR System (ABI, USA) was employed. The primers used in this study were as follows, for β-actin: ACTCGTCATACTCCTGCT and GAAACTACCTTCAACTCC and for HMGB1: ATATGGCAAAAGCGGACAAG and GCAACATCACCAATGGACAG.

The secreting HMGB1 measurements

The cells were transfected with NP1 plasmid. Then 100 μL of cell supernatant was collected at different time points by centrifugation at 5000 rpm for 10 min to remove the cell debris. Subsequently, supernatant was taken for analysis. The HMGB1 ELISA Kit (Mlbio, China) was used to detect HMGB1 according to the manufacturer's protocol. The standard curve of HMGB1 was used to calculate the protein levels (pg/mL).

Statistical analysis

The result was expressed by means of S mean+standard deviation. The $P\leq0.05$ showed statistical difference.

RESULTS

NP1 does not induce apoptosis of A549 cells, but affects cell viability

NP1 is a non structural protein of HBoV1 which was found to cause apoptosis of HeLa cells. To determine whether the NP1 causes the apoptosis of A549 cells, the apoptosis assay was performed using Tunel apoptosis detection (FITC) Kit. No apoptosis was observed in the cells upon NP1 expression plasmid transfection, indicating that NP1 could not induce apoptosis in A549 cells (*Figure 1A*). We also detected the expression of caspase 3. After expression of NP1 in A549 cells, Caspase3 was not activated (*Figure 1B*). To detect the possible impacts of NP1 on cell migration, we performed a cell scratch test. After 12h of NP1 expression, the scratch area was obviously wider in NP1-expressed A549 cells than in untreated cells (*Figure 2A*). To investigate whether the slowdown of cell migration was related to cell viability, CCK8 assay was used to detect the impacts of NP1 on the viability of A549 cells transfected with NP1 plasmid 48h post transfection (*Figure 2B*). The results showed that A549 cell viability was down-regulated by NP1.

NP1 promotes cell autophagy

To test whether autophagy occurs in NP1 expressing A549 cells, the western blot assay was performed to detect autophage-related proteins. The expression levels of autophagy-related proteins p62, LC3-II, and beclin 1 were measured in this study. The results showed that, compared to mock-transfected A549 cells, NP1 expressed A549 cells exhibited the obviously higher expression levels of beclin-1 and LC3-II (*Figure 3A*). However, p62 level was significantly down-regulated in A549 cells transfected with NP1 gene, suggesting that autophagy but not apoptosis occurred due to the expression of NP1 in A549 cells.

NP1 activates Phosphorylation of STAT3

Autophagy was mediated by some key signal molecules of cellular pathways (e.g., STAT3, MAPKs and PI3k/AKT/mTOR) (Mancias and Kimmelman, 2016). To determine whether the NP1 trigger autophagy in A549 cell and which pathway is involved, the expression of phosphorylated STAT3 and PRKA was analysed. Our results revealed that the expression of phosphorylated STAT3 was up-regulated

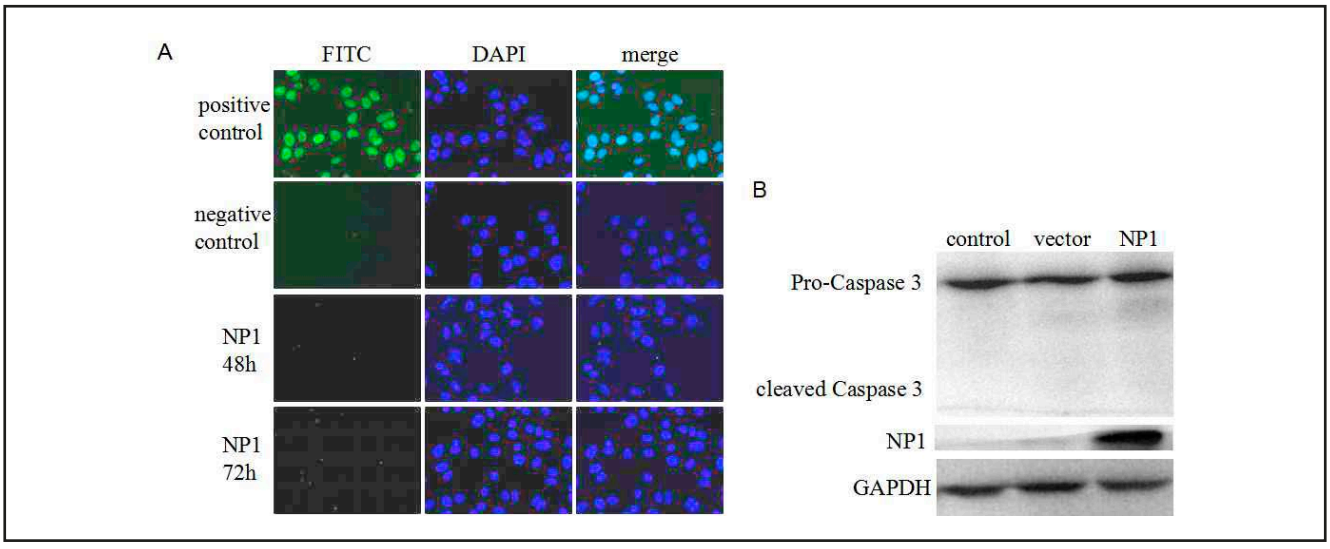

Figure 1 - *NP1 did not induce apoptosis in A549 cells. The A549 cells were transfected with pCMV-NP1 plasmid. (A) At the indicated time points, the cells were fixed and labeled with the FITC and then analyzed with fluorescence microscope. The late apoptotic population was recognized as apoptotic cells. (B) After 48h of transfection, cellular total proteins were separated by 10% SDS-PAGE and. the western blot assay was performed to detect caspase 3. No cleavage of the caspase 3 was observed. The GAPDH was used as an internal control.*

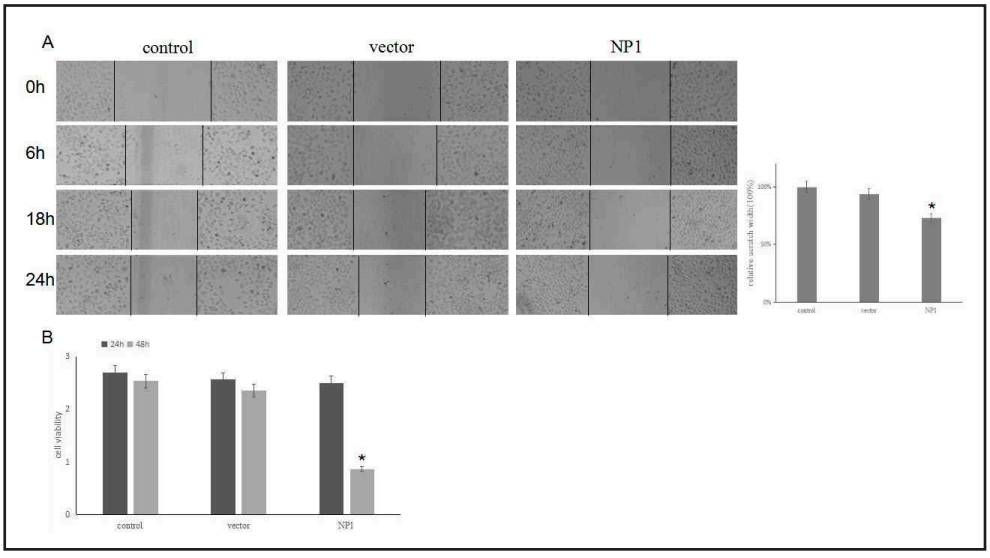

Figure 2 - *P1 expression suppressed the A549 cell migration and viability. (A) pCMV-NP1 was transfected into A549 cells. Then a cell scratch test was performed to detect the cell migration after 6h, 18h, 24h. (B) A549 cells were stained with CCK-8 after 24 and 48 h of transfection with pCMV-NP1. *P<0.05.*

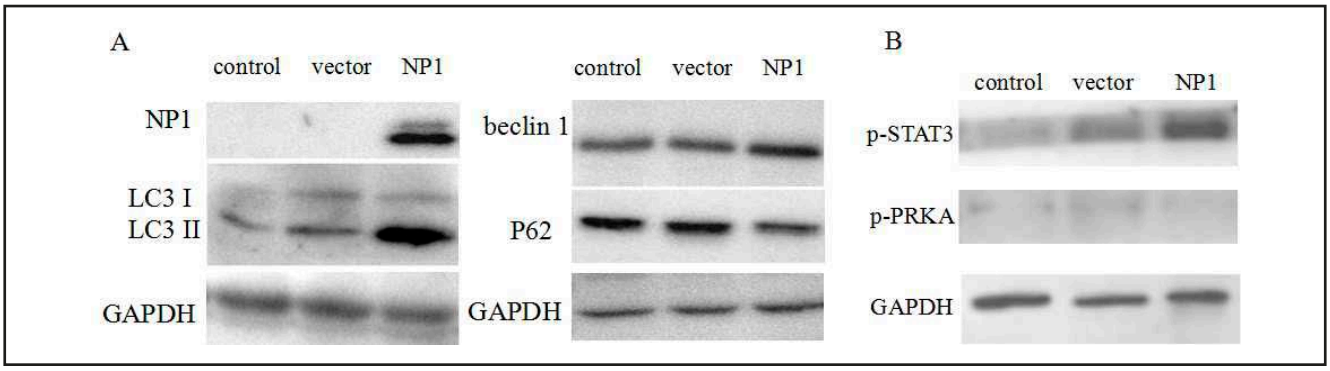

Figure 3 - *NP1 promoted cell autophagy through STAT3 signaling pathway in A549 cells. pCMV-NP1 was transfected into A549 cells. After 48h of transfection, cell protein was extracted. The western blot assay was performed to detect autophage-related proteins (A), phosphorylated STAT3 and PRKA (B).*

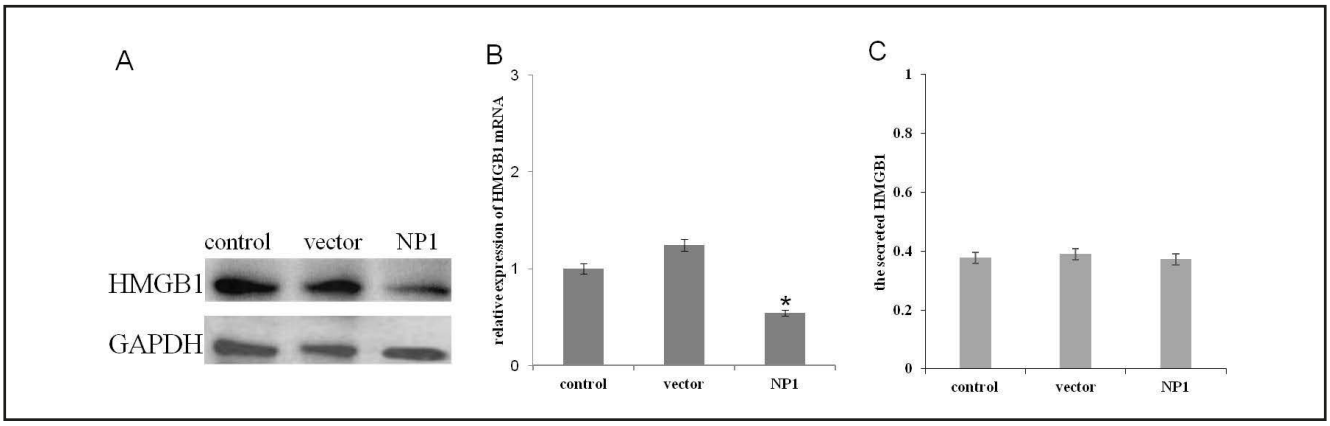

Figure 4 - *NP1 suppressed the expression of HMGB1 in A549 cell. (A) pCMV-NP1 was transfected into A549 cells. After 48h of transfection, cell samples were collected to detect HMGB1 expression by Western Blot as described in the methods and GAPDH was served as an internal control. (B) pCMV-NP1 was transfected into A549 cells. The total RNA of cells was extracted 24 h post transfection. The expression of HMGB1 mRNA was quantified by qRT-PCR. (C) The A549 cells were transfected with pCMV-NP1 plasmid. Then 100μL of cell supernatant was collected by centrifugation to remove the cell debris. The secreted HMGB1 in supernatant was detected by ELISA Kit. *P<0.05.*

by NP1 (*Figure 3B*). This data suggested that NP1 induced autophagy in A549 cells through STAT3 signaling pathway.

NP1 suppresses the expression of HMGB1 in A549 cell

HMGB1 was highly expressed in A549 cells. Zhang et al (Zhang *et al*., 2015) reported that HMGB1 played an important role in the development of lung cancer. In order to investigate whether NP1 affects the expression of HMGB1, the A549 lung cancer cells were transfected with NP1 plasmid. As shown in *Figure 4*, the HMGB1 levels were significantly lower 48 h post transfection with NP1 construct. ELISA was also used to detect whether secreted HMGB1 was found in cell culture supernatant. The results showed that no HMGB1 was detected in the cell culture supernatant, indicating that the decreased level of HMGB1 within the cell was not due to the extracellular secretion of HMGB1 (*Figure 4*). The HMGB1 expression presented a down-regulation in NP1-expressed cell, suggesting NP1 could inhibit endogenous expression of HMGB1 which may be associated with cell death.

DISCUSSION

HBoV1 is one of the most important pathogens of respiratory tract infections in children (Qiu *et al*., 2017). Since it was identified in 2005, HBoV1 has attracted wide attention because of its high potential for development as a gene therapy vector (Yan *et al*., 2013). However, the mechanism of HBoV1 infection has not been fully understood. NP1 is a nonstructural protein of HBoV1 and plays an important role in viral DNA replication. Some studies have shown that parvovirus can induce apoptosis or necrosis of cells (Chen *et al.*, 2010) and NP1 of HBoV1 induced apoptosis of HeLa cells (Sun *et al*., 2013). In this study, we demonstrated that NP1 of HBoV1 can induce autophagy instead of apoptosis in A549 cells which derived from the respiratory system. This strategy might contribute to the replication and proliferation of the virus in the lung, while HeLa cells come from the cervix which was not the specific targeted organ of HBoV1. As a multifunctional protein, NP1 plays multifarious roles in different cells, which just reflects the tissue or organ specific of HBoV1 infection.

In most cases, after the virus infects the host cells, it leads to the death of the host cells, including necrosis, apoptosis and pyroptosis. Deng et al. (Deng *et al.*, 2017b) reported that HBoV1 killed airway epithelial cells by inhibiting apoptosis, thus promoting pyroptosis. This strategy seems to facilitate HBoV1 replication. The relationship between autophagy and viral infection has been extensively studied in a number of viruses. Because autophagy plays an important role in different cellular functions, many viruses use autophagy mechanism to facilitate their own replication and survival. On the other hand, the autophagy pathway has become an important effector of antiviral immunity (Dong and Levine, 2013; Jackson, 2015; Lennemann and Coyne, 2015). Viruses produce a large number of viral components in the process of infection, such as DNA, RNA and viral proteins. DENG et al (Deng et al., 2017c) confirmed that HBoV recombinant expressing vector (pWHL-1) induced apoptosis and autophagy in HBECs. This phenomenon may be accomplished by the co-participation of different non-structural proteins, including NS (1-3)(Deng *et al.*, 2017a) and NP.

Viral proteins play an important role in inducing autophagy. The co-expression of 2BC and 3A proteins of poliovirus could induce the formation of bilayer membrane autophages, similar to the autophagic vesicles observed under electronic microscopy upon poliovirus infection. The expression of 2BC protein can induce the conversion of LC3. The expression of 3A protein can cause ER swelling, but it can not induce vesicle formation and LC3 conversion (Taylor and Kirkegaard, 2007) .

So far, several viral proteins have been found to specifically induce autophagy signals, including RTA transcription factor of EBV, X protein of hepatitis B virus, T antigen of SV40 and ns4b, NS5A and NS5B proteins of HCV (Jackson, 2015; Paul and Munz, 2016). In this study, NP1 protein expression alone could up-regulate Beclin1 and LC3 II, but down-regulate the expression of P62 and HMGB1 in A549 cells. Lipidated LC3-II serves as a reliable marker of autophagy. Beclin 1 is involved in the early stage of autophagic vesicle formation. P62 is a substrate degraded by autophagy, often acting as a useful marker for inducing autophagy (Lv and Lu, 2017). HMGB1,

which has extracellular functions in inflammation and cancer progression, plays a critical role in cancer metastasis (Chen *et al*., 2018; Liu *et al*., 2019). High levels of HMGB1 are always observed in various cancer types, including breast, gastric, and lung cancers (Hu *et al.*, 2018; Wu *et al*., 2019). HMGB1 can promote the migration and invasion of lung cancer cells (Wang *et al*., 2018). In the present study, we demonstrated that NP1 significantly inhibited the level of HMGB1. Therefore, NP1 has potential to be used as an inhibitor of HMGB1 to control the growth of lung tumor.

Some studies have shown that a variety of viruses could trigger autophagy mediated by GTPase family proteins. These results confirm that multiple viruses have selectively evolved to induce autophagy through signaling pathways. Simvastatin and metformin enhance PTEN and autophagy by mTOR and inhibit the growth of hepatitis C virus infected cells (Del Campo *et al*., 2018). Rotavirus encoded virus like small RNA triggers autophagy through PI3K/Akt/mTOR pathway targeting IGF1R (Zhou *et al*., 2018). In this study, NP1 induced A549 autophagy was activated by STAT3 signaling pathway. However, the signal pathway of NP1 regulating autophagy needs further study.

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