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HTNV-induced upregulation of miR-146a in HUVECs promotes viral infection by modulating pro-inflammatory cytokine release

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ABSTRACT

Increasing research has shown a link between viruses and miRNAs, such as miRNA-146a, in regulating virus infection and replication. In the current study, the association between miR-146a and hantaan virus (HTNV) infection in human umbilical vein endothelial cells (HUVECs) was investigated, with a focus on examining the expression of pro-inflammatory cytokines. The results showed that HTNV infection promoted the production of miR-146a in HUVECs and activated nuclear factor-κB (NF-κB) signaling, along with the upregulation of pro-inflammatory cytokines, including interleukin 8 (IL-8), C-C Motif Chemokine Ligand 5 (CCL5, also RANTES), interferon-inducible protein-10 (IP-10) and interferon beta (IFN-β). Moreover, miR-146a exhibited a negative regulatory effect on the NF-κB pathway. Accordingly, a miR-146a inhibitor increased the expression of IL-8, CCL5, IP-10 and IFN-β, whereas a miR-146a mimic reduced the levels of these cytokines. Consequently, exogenous transduction of miR-146a significantly enhanced HTNV replication in HUVEC cells. We also discovered that viral proteins (NP/GP) contributed to miR-146a expression via enhancement the activity of miR-146a promoter. In conclusion, these results imply the negative regulation of miR-146a on the production of HTNV-induced pro-inflammatory cytokines contributes to virus replication, which suggest that miR-146a may be regarded as a novel therapeutic target for HTNV infection.

1. Introduction

Hantavirus, a single-strand negative RNA virus, belongs to the *Bunyaviridae* family and is a globally distributed animal-derived virus [1]. The hantavirus genome consists of three segments, designated as S (small), M (middle), and L (large), encoding the nucleocapsid protein (NP), envelope glycoproteins G1 and G2, and virus RNA polymerase respectively [2]. Pathogenic hantavirus infection in humans mainly causes two types of disease: hantavirus cardiopulmonary syndrome (HCPS), and hemorrhagic fever with renal syndrome (HFRS) [3]. Hantaan virus(HTNV) and Seoul virus(SEOV) are the most pathogenic hantavirus species found in Asia, with mortality of 5–10% [4].

Hantavirus primarily infects human vascular endothelial cells and induces dysfunction in capillaries and small vessels [5,6]. Although the pathogenic mechanism of hantavirus is not completely clear, it is known that the immune response plays an important role in hantavirus-induced disruption of the endothelial barrier, especially redundant immune response [7]. The NF-κB family is a pivotal transcription factor in regulating the innate and

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adaptive immune response to virus infection. After activation, NF-κB protein is transported into the nucleus to induce the transcription of immune-response genes, such as I-IFN and pro/anti-inflammatory cytokines [8]. Yu et al. demonstrate that the nuclear translocation of interferon regulatory factor 3 (IRF-3) and NF-κB occurs in the HTNV-infected human vascular endothelial cells (EVC-304) rather than toll-like receptor 4 (TLR4)-knockout cells, which suggests that HTNV infection triggers TLR4-mediated activation of IRF3 and NF-κB via a myeloid differentiation factor 88 (MyD88)-independent signaling pathway of the innate immune responses [9]. Another study shows that HTNV induces NF-kB activation immediately after infection and the serine/threonine kinase (Akt)/NF-kB pathway is involved in the expression of cytokines/chemokines and adhesion molecules in HTNV-infected HUVECs, which contributed to the vascular permeability and pathogenesis of HFRS [10]. Considering that direct cytopathic effects of pathogenic hantaviruses have not been observed, the increased vascular permeability might be caused by these unbalanced immune response triggered by viral infection. Therefore, it is necessary to explore how host cells accurately control and balance these antiviral and inflammatory response, especially the functional regulation of the disease-related immune regulatory factors.

MicroRNAs (miRNA) belong to the small non-coding RNAs and regulate the expression of gene at a post-transcriptional level, which have been shown to be related to various viral infections [11]. In the process of discovering miRNAs that affect host response to microbial infection, miR-146a was first defined as an immune system modulator [12]. NF-κB activation has been shown to upregulate miR-146a expression and in turn, miR-146a functions as a negative feedback regulator by targeting two key adapter proteins, interleukin-1 receptor-related kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) [13]. miR-146a has been reported to play a vital role in regulating viral infection and replication. Hepatitis C virus (HCV) infection can enhance miR-146a expression in different cell-based model for HCV infection, as well as in liver tissue from HCV patients, and promotes the production of infectious HCV particles *in vitro*, which is conducive to pathogenesis of liver disease [14]. It has been suggested that enterovirus 71 (EV71)-induced miR-146a is involved in viral pathogenesis by blocking NF-κB signaling and suppressing I-IFN expression, and thus inhibition of miR-146a reduces mortality in mice by restoring I-IFN production [15]. MicroRNA array results suggest that pathogenic HTNV, rather than nonpathogenic prospect hill virus (PHV), induces high expression level of miR-146a in HUVECs, instead of in the epithelial or macrophage cells [16]. However, the functional involvement of miR-146a in HTNV-mediated endothelial pathogenesis remains to be elucidated.

This study was conducted to investigate the relationship between miR-146a and HTNV. The involvement of miR-146a in HTNV infection-induced upregulation of pro-inflammatory cytokines, including IL-8, CCL5, IP-10, and IFN-β, in HUVECs was also investigated.

2. Materials and methods

2.1. Cells and virus

HUVECs were purchased from ScienCell Research Laboratories (Cat No: 8000, Carlsbad, USA) and cultured in ECM Medium (Cat No:1001, ScienCell). HUVECs were cultured in poly-L-lysine-coated flasks and used for no more than six passages. HTNV 76–118 was obtained from the Institute of Virology, Chinese Center for Disease Control and Prevention (CDC, Beijing, China). The Reed-Muench method was used to determine hantaviral $TCID_{50}$ by indirect immunofluorescence assay.

2.2. RNA extraction and quantitative real-time PCR (qPCR) analysis

TRIZOL (Invitrogen, USA) was employed to extract total cellular RNA from cultured cells under the manufacturer's instructions. Complementary DNA was synthesized following the procedure described below using random primers and reverse transcriptase (M-MLV, Promega): 70 °C for 5 min, 25 °C for 15 min, 37 °C for 60 min, and 94 °C for 10 min. Special stem-loop primers were subjected to miR-146a and U6 RT reactions based on previous reports [17]. Real-time PCR was done using a master mix for SYBR Green qPCR (Toyobo, Japan) in a CFX96 Real-Time System with the following conditions: 95 °C for 3 min, and 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. The primers used for the experiment were showed in Table 1. We calculated the relative expression level of each gene with the $2^{-\Delta\Delta CT}$ method as described by Schmittgen and Livak [18].

2.3. Western-blotting

The RIPA-extracted cell lysates were resuspended with SDS lysis buffer and inactivated at 95 °C for 5 min, followed carrying out the protein experiments based on previous reports [19]. The primary antibodies used in the experiments were as follows: HTNV 76–118 strain monoclonal antibody (Abnova, MAB5482, Taiwan), NF-kB p65 polyclonal antibody (Abcam, ab16502, Cambridge, USA) and GAPDH monoclonal antibody (Tianjin Sungene Biotech DKM9002T, Beijing, China). The secondary antibodies used for western-blotting were as follows: anti-rabbit IgG (CST7074S, USA) and anti-mouse IgG (BA1050, Wuhan, China).

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for IFN-β (Cusabio, Wuhan, China), IL-8, IP-10, and CCL5 (4A Biotech, Beijing, China) were used to detect the levels of each cytokine in the supernatants derived from HTNV-infected HUVECs. The supernatant samples were diluted ten-fold and the kits were applied according to the instructions. Standard curves were generated based on the results of the standard samples provided by the manufacturer. The cytokine levels were determined from the standard curve.

2.5. miR-146a inhibitor/mimic and transfection

The antisense miR146a oligonucleotide (miR146a inhibitor) and its negative control (iNC) were designed based on a previous report [20] as follows: 5′-AACCCAUGGAAUUCAGUUCUCA-3' (single-stranded oligo) and 5′-CAGUACUUUUGUGUAGUACAA-3' (iNC). The miR-146a mimic (dsRNA oligos), miRNA mimic control (mNC), inhibitor of miR-146a, and iNC were ordered from Ribobio (Guangzhou, China). HUVECs were cultured into 24-well plates (1.0×10^5 cells/well) and transfected with the miR146a mimic or inhibitor using Lipo2000 (Invitrogen, CA, Carlsbad, USA) according to the manufacturer's protocol. HUVECs were then cultured for two

Table 1
qRT-PCR primers and sequences.

gene	Sense(5′–3′)	Antisense(5′–3′)
HTNV	TCTAGTTGTATCCCCATCGACTG	ACATGCGGAATACAATTATGGC
P65	CTGCAGTTTGATGATGAAGA	TAGGCGAGTTATAGCCTCAG
IFNB1	CGCCGCATTGACCATCTA	GACATTAGCCAGGAGGTTCTCA
CCL5	GTAAGAAAAGCAGCAGGCGG	GACACCACACCCTGCTGCT
IP10	CTACACCAGTGGCAACTGCT	TCCCGAACCCATTTCTTCT
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT