Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
miR-146a-5p promotes replication of infectious bronchitis virus by targeting IRAK2 and TNFRSF18

Hui Liu¹, Xin Yang¹, Zhi-kun Zhang, Wen-cheng Zou, Hong-ning Wang∗

Key Laboratory of Bio-Resource and Eco-Environment of Ministry of Education, Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, College of Life Sciences, Sichuan University, Chengdu 610065, Sichuan, PR China

A R T I C L E   I N F O

Keywords:
IBV
miR-146a-5p
IRAK2
TNFRSF18

A B S T R A C T

Avian infectious bronchitis virus (IBV) is a coronavirus which infects chickens (Gallus gallus) of all ages and causes significant economic losses to the poultry industry worldwide. The present study aims to analyze the miRNAs related to pathogenicity of nephropathogenic IBVs. It was found that four miRNAs (miR-1454, miR-3538, miR-146a-5p and miR-215-5p) were related to the infection of virulent nephropathogenic IBV with transcript per million (TPM) > 500 and more than a 2-fold alteration. In vitro study results showed that the alterations of these four miRNAs were consistent with in vivo data. In vitro, we found that high levels of miR-146a-5p could enhance the replication of IBV at the early stage of infection, and its down regulated level could slow down the replication of IBV. Finally, high levels of exogenous miR-146a-5p in HD11 cells led to down regulation of IL-1 receptor associated kinase-2 (IRAK2) and Tumor necrosis factor receptor superfamily member 18 (TNFRSF18) genes. Luciferase reporter assays revealed that miR-146a-5p could bind to the 3′-UTRs of IRAK2 and TNFRSF18. This is the first study demonstrating that IBV induced miR-146a-5p is related to virus pathogenesis by down regulating IRAK2 and TNFRSF18, which may serve as a therapeutic strategy for the prevention of IBV infections.

1. Introduction

Avian infectious bronchitis virus (IBV) is an enveloped, positive-strand RNA virus of Gamma coronavirus which infects chickens. It causes avian infectious bronchitis (IB), which is an acute highly contagious disease with severe economic losses in poultry industry worldwide [1]. IBV has a large number of genotypes and in China, 19 strains are isolated during 2011–2012. Infected chickens develop respiratory symptoms, coughing, sneezing, depression, nasal discharge, kidney, gonads, reduced egg production, poor egg quality and death. In recent year, nephropathogenic IBVs have become prevalent genotypes in China [2], new nephropathogenic isolates induce more severe lesions to kidney and show high mortality in young chickens [3]. Currently only IBV Beaudette strain can replicate in HD11 [4] or Vero [5] cell lines. Several recent works have proved the induction of apoptosis and suppression of immune response are related to the pathogenicity of nephropathogenic IBVs [6,7]. However, the key factor for these process during infection are still unclear.

Transcriptome analysis of chicken kidney infected with IBV identify that differentially expressed genes are involved in signal transduction, cell adhesion, immune responses, apoptosis regulation, positive regulation of the I-kappaB kinase/NF-kappaB cascade and cytokine stimulus [8]. The mRNAs are mostly supposed to be regulated by miRNAs, which are a class of small non-coding RNAs that suppress the expression of target genes by binding to the 3′-untranslated regions (UTRs) [9]. Post infection of IBVs can encode miRNAs or influence the expression levels of cellular miRNAs [10,11]. miRNAs have been reported to play an important role in virus pathogenesis such as miR-122, which may be able to stabilize the HCV genomic RNA [12], and the immune response related miRNAs such as miR-130a, miR-155, miR-23b miR-146a-5p which can help the virus to augment its replication or in evasion of cellular immune response [13–15].

However, few studies have reported about the miRNA alterations in the post infection of IBV. In our previous study, we have described the transcriptome of chicken kidneys infected with nephropathogenic IBVs at mRNA and miRNA level. Chicken kidney infected with three nephropathogenic IBV with different virulence revealed the differentially expressed genes and miRNAs [6,16]. Among them, most of the differentially expressed (DE) miRNAs are related to immune response, cell apoptosis, DNA replication and metabolic pathways [6]. 58 differentially expressed miRNAs are considered responsible for the differentially expressed (DE) miRNAs. As we described several of these miRNAs

¹ Corresponding author. Tel./fax: +86 028 8547 1599.
E-mail address: whongning@163.com (H.-n. Wang).
² Hui Liu and Xin Yang have contributed equally to this work.

https://doi.org/10.1016/j.micpath.2018.04.046
Received 30 January 2018; Received in revised form 20 April 2018; Accepted 23 April 2018
Available online 24 April 2018
0882-4010/ © 2018 Elsevier Ltd. All rights reserved.
are in accordance with the virus titers in each group, revealing that they may serve as determinants of the pathogenicity of nephropathogenic IBVs.

The present study aims to investigate the role of abundant DE miRNAs in HD11 cells infected with Beaudette IBV strain. Fortunately, we found the level of miR-146a-5p is related to the replication of IBV at the early stage of infection. miR-146a-5p is well known immune response gene, overexpression of miR-146a-5p leads to the suppression of cellular inflammatory response and decrease in cytokine secretion [17]. Up regulations of miR-146a-5p have been reported in the infection of DENV, CHIKV, VSV, AIV (H3N2), and HCV [18]. This is the first study described the role of miR-146a-5p during the replication of IBV.

2. Materials and methods

2.1. Cell lines and virus strain

The IBV strain Beaudette was kindly provided by Prof. Ding-xiang Liu, Nanyang Technological University. The LDT3-A, SCK2 and SCDY2 were nephropathogenic IBV strains and stored by the Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province [6]. The Vero and HEK 293T cell line was procured from the American type culture collection (ATCC CCL81). The HD11 cell line was kindly provided by Prof. Xin-An Jiao, Yang Zhou University. Both cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) (HyClone) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

2.2. Virus infection and miRNA alterations in vitro

HD11 and Vero cells were seeded at a density of 60–70% per well in 6-well plates. The cell lines were then inoculated with Beaudette at 10^3.242 TCID50, HD11 and 10^2.653 TCID50, Vero and incubated at 37 °C (5% CO_2) for 1 h. Subsequently, the cells were washed with PBS and cultured in 37 °C incubator with DMEM supplemented with 2% FBS (5% CO_2) for 24 h. Total RNA of each well was isolated by TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, and reverse transcript by miRNA specific RT primers (Ribobio). Virus titration in cells were calculated every 4 h of post infection with real-time fluoroscent quantitative PCR by 3'-UTR primers (5'-ACAGGTTCCTGGTGTTGTTTAGTA-3'; 5'-AGTGTTTCGGGATGCGTCTTT G-3') [19], using β-actin as reference gene. The expression levels of miRNA-146a in cells at each time point was also calculated with loop PCR, using U6 as reference miRNA (Bulge-loop primers are shown in Supplementary Table 1).

2.3. miRNA mimics and inhibitors transfection

miRNA mimics and inhibitors of miRNAs and control mimics were synthesized by Ribobio (China) and diluted to 50 nM. Cells in 6-well plates were transfected with miRNAs with lipofectamine 2000 (Invitrogen) according to the manufacturer instruction. 12 h after transfection, cells were incubated with Beaudette at 10^3.242 TCID 50, incubated for 1 h at 37 °C (5% CO_2). The cells were washed with PBS and cultured in 37 °C incubator with DMEM supplemented with 2% FBS (5% CO_2) for 24 h. Virus titers and miRNA levels were detected by real-time PCR. In addition, the virus titers in HD11 cells transfected with miR-146a-5p and control mimics were tested every 4 h as described above to build growth curves.

2.4. Quantitation of predicted genes

Potential target miRNAs for miRNA-146a that expressed differently in the sequencing results were analyzed by miRanda. The miRNA and miRNA libraries used in this study were identified in our previous study [6,16]. miRNA expression of predicted genes in HD11 cells after transfection, mock-infected or transfection-infected lung tissues were assayed at 0, 12, 24 and 48 h of post infection. The miRNA levels of miR-146a-5p was evaluated at each time point. Total RNA of each well was isolated by TRizol Reagent (Invitrogen, USA), and reverse transcript by random primers. Transcription levels of the predicted genes were amplified by primers (Supplementary Table 2) and detected by SYBRGreenII (Takara, China). The cytopathic time of each group was observed by inverted microscope.

2.5. Dual-luciferase activity assay

PmirGLO vector was utilized to analyze the binding activities of miR-146a-5p to the predicted sites in the 3'-UTR of each gene. The genes containing the predicted 3'-UTR binding sites (TNFRSF18 and IRAK2) were amplified by PrimerSTAR GXL DNA Polymerase (TaKaRa), and cloned into pmiRGLO at the downstream multiple cloning site of luc2 (primers are listed in Supplementary Table 3).

HEK 293T cells were cultured in 6-well plates at a density of 60–70%. The constructed plasmids were transfected or co-transfected with miRNA mimics or inhibitors into HEK 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. After 24 h of post transfection, firefly and Renilla luciferase activities were measured by thermo scientific microplate reader.

2.6. Statistical analysis

All the transfection experiments and RT-qPCR experiments were performed in triplicate. Data from each independent experiment was shown as mean value with standard deviation. Statistical analysis was estimated by 2^-ΔΔCT method, p < 0.01 (**) were considered statistically significant.

3. Results

3.1. Alteration of miRNAs after IBV infection in vivo

MicroRNAs have been reported to involve in the virus infection [20]. miRNA and mRNA sequencing analysis in chicken kidney tissues infected with IBV results showed that the expression levels of several miRNAs were in consistent with the virus titers and lesions in chicken kidney [16]. In this study, we screened and selected 4 miRNAs (miR-1454, miR-3538, miR-146a-5p, miR-215-5p) with TPM > 500 and more than a 2-fold alterations (Supplementary Table 4) [16]. These miRNAs were predicted to play an important role in IBV pathogenesis in vivo.

3.2. Up-regulation of miR-146a-5p in cells infected with Beaudette

To validate the regulation of miRNAs in cells infected with IBV, the expression levels of miR-1454, miR-3538, miR-146a-5p, miR-215-5p in HD11 and Vero cells infected with Beaudette were determined by qPCR. As shown in Fig. 1, miR-146a-5p was significantly (p < 0.01) up regulated at early stage at 8 h, at 24 h it was increased up to 2-fold in both infected cell lines when compared to control. These changes may lead to the forming of cytopathic effect (CPE) (Fig. 1). The expression level of miR-146a-5p may directly regulated by cells in anti-viral progress, or regulated by virus replication.

miR-146a-5p role in the replication of IBV at the early stage of infection.

To determine the role of these miR-1454, miR-3538, miR-146a-5p, miR-215-5p in the lifecycle of IBV infected cells, we examined these miRNAs role in replication of virus in HD11 cells. It was found that the high level of miR-146a-5p could promote the replication of Beaudette in HD11 cells (Fig. 2a). In addition, we tested the growth curve of Beaudette in HD11 cells in the post transfection of miR-146a. As shown in Fig. 2b & c, high levels of miR-146a-5p can promote the replication
of Beaudette at the early stage post infection, subsequently it was increased to 4-fold of virus genome at 8 h period. The virus titer peaked at 32 h, and the time of cytopathic effects reduced to 33 h when compared to 36 h of untreated cells (control). These results indicating that cytopathic effect is mainly related with the virus titers in cells and further confirmed the promotion of replication of IBV.

3.3. miR-146a-5p related genes

Transcriptome and target gene analysis results displayed 47 related genes which may be regulated by miR-146a. By annotation, 8 predicted target genes (IRAK2, LRP2, ELK4, TAPBPL, TNFRSF18, TGFBI, CCL19, MVB12B) were involved in regulation of immune responses and cell apoptosis, which may be related to the multiplication of IBV. Hence, we investigated the expression levels of these 8 genes (Fig. 3a) in HD11 and Vero cells transfected with miR-146a-5p mimics (Fig. 3a). Expression analysis results showed the down regulations of TNFRSF18 and IRAK2 compared to control cells. Further, to verify the target sequences of miR-146a, the 3′-UTRs 22bp fragments of TNFRSF18 and IRAK2 containing the predicted binding sites (Fig. 3b) were cloned into pmirGLO vector. As shown in Fig. 3c, miR-146a-5p mimic could inhibit the luciferase activity of the reporter vector of TNFRSF18 and IRAK2, but not the ones with mutated binding sites (Fig. 3c). These data indicating that TNFRSF18 and IRAK2 are the main targets of miR-146a-5p to promote the replication of IBV.

4. Discussion

IBV was first reported to cause infectious bronchitis and respiratory diseases in chicken in the early 1930s and the virus can spread to many other epithelial cells post infection. Kidney is not a primary target for the infection of IBV, but new isolates of recent years are more virulence with kidney lesions [21]. A study of differences of dynamic distribution between nephropathogenic infectious bronchitis virus (IBV) strains such as SAIBK, M41 and H120 in SPF chicken exhibited that the kidney and the lung were the most sensitive organs in IBV infection [22]. Our in vitro study results exhibited that the miR-146a-5p was associated with the rapid multiplication of virus at the early stage of infection by down regulating the TNFRSF18 and IRAK2. In addition, regulation of miR-1454, miR-3538, miR-146a-5p, miR-215-5p were consistent with in vivo data in HD11 cells. This result revealed the role of these 4 miRNAs in replication of IBV.

After IBV infection, the host can alter the miRNA levels in the cells to activate antiviral effectors. However, several studies have reported that the virus can make use of cellular to enhance the infection, such as the 5′-UTR of HCV binds to miR-122, the 3′-NTR of bovine viral diarrhea virus (BVDV) binds to miR-17 and let-7c. These binding sites have been exhibited the increasing stability and translation of the viral RNA [20]. On the other hand, the virus can change the expression level of cellular miRNAs, which may be beneficial to viral infection. Several miRNAs have been reported to involving in the down regulation of IFN-α/β, which may benefit the infection and multiplication of virus. Such as miR-758, miR-373, miR-30e and miR-146a. The up regulation miR-146a-5p have been proved to be induced by latent membrane protein in Human T-cell leukemia virus type 1 (HTLV-1), Human immunodeficiency virus (HIV), Vesicular stomatitis virus (VSV), Epstein-Barr virus (EBV) and K13 protein in Kaposi’s sarcoma-associated herpesvirus (KSHV) [23,24]. The key viral proteins in the regulation of miR-146a need to be further investigated.

miR-146a-5p is a negative inflammatory regulatory factor, over-expression of miR-146a-5p could promote the replication of dengue virus and Hendra virus [23,25]. It mainly involved in Toll-like receptor...
helpful to make a new strategy for the control of IBV. In the post-infection periods [27]. Hence, this study results could be induced in animal experiment results revealed that up-regulation of IL15 may only in TLR3 pathway, and resulting in the up-regulation of IL15. However, up-regulated immune response associated genes were mainly enriched Further, the protein-protein interaction (PPI) analysis showed that the results exhibited that the NF-κB signaling pathway and downstream proinflammatory chemokine. In VSV model, it targets TNF receptor-associated factor 6 (TRAF6), IL-1 receptor associated kinase-1 (IRAK1) and IRAK 2 genes. In the present study, we described IRAK 2 as a target for miR-146a-5p in IBV model. In addition, for the first time it was found that TNFRSF18 was a target of miR-146a. However, the expression level of TNFRSF18 was relatively low, the function of TNFRSF18 is need to be further characterization. On the other hand, we found Low density lipoprotein receptor-related protein 2 (LRP2) was down regulated by miR-146a-5p during the infection of virulent IBV. Down regulation of LRP2 has been reported leading to cell apoptosis in Alzheimer’s disease. Similarly, miR-146a-5p was also reported target in enterovirus 71-induced cell apoptosis. These indicated that miR-146a-5p play an important role in the pathogenesis of IBV.

IL-1R-associated kinase 2 (IRAK2) is required for IL-1R-induced NF-κB activation in mammals [26]. Transcriptome sequencing analysis results exhibited that the NF-κB1a and NF-κB1z were up-regulated. Further, the protein-protein interaction (PPI) analysis showed that the up-regulated immune response associated genes were mainly enriched in TLR3 pathway, and resulting in the up-regulation of IL15. However, animal experiment results revealed that up-regulation of IL15 may only induce inflammation, but not in resistance of IBV. In this study, we found that miR-146a-5p increased the replication of IBV by induced down-regulation of IRAK2. IRAK2 is also involved in TLR signaling and type I interferon production. Thus, we infer that IRAK2 associated RIG-I-dependent type I IFN production may effective in suppression of IBV in the post infection periods [27]. Hence, this study results could be helpful to make a new strategy for the control of IBV.

Taken together, this is the first study revealed that the IBV induced the up-regulation of miR-146a-5p in vitro in accordance with in vivo sequencing data. In addition, our studies revealed that miR-146a-5p could serve as a key factor in the pathogenesis of IBV.

Conflicts of interest

All authors declare that they have no conflict of interest.

Ethical approval

All authors have seen the manuscript and approved to submit to "Microbial Pathogenesis".

Acknowledgments

This research was supported by the National Key R&D Program of China (2017YFD0500703), State Natural Sciences Foundation (31302094, 31372442), the Program of Main Livestock Standardized Breeding Technology Research and Demonstration (2016NYZ0052), the Project for Science and Technology Support Program of Sichuan Province (2014NZ0002, 2016NZ0003) and the China Agriculture Research System (CARS-40) National System for Layer Production Technology (CARS-40-K14).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2018.04.046.

References

[1] D. Cavanagh, Coronavirus avian infectious bronchitis virus, Vet. Res. 38 (2007) 281–297.
[2] Z. Zhang, Y. Zhou, H. Wang, F. Zeng, X. Yang, Y. Zhang, et al., Molecular detection and smoothing spline clustering of the IBV strains detected in China during 2011-2012, Virus Res. 211 (2016) 145–156.
[3] X. Wu, X. Yang, P. Xu, L. Zhou, Z. Zhang, H. Wang, Genome sequence and origin analyses of the recombinant novel IBV virulent isolate SAIBK2, Virus Gene. 52 (2016) 509–520.
[4] X. Han, Y. Tian, R. Guan, W. Gao, X. Yang, L. Zhou, et al., Infectious bronchitis virus infection induces apoptosis during replication in chicken macrophage HD11 cells, Viruses 9 (2017) 198.
[5] T.S. Fung, Y. Liao, D.X. Liu, The endoplasmic reticulum stress sensor IRE1α protects cells from apoptosis induced by the coronavirus infectious bronchitis virus, J. Virol. 88 (2014) 12752–12764.
[6] H. Liu, X. Yang, Z. Zhang, J. Li, W. Zou, F. Zeng, et al., Comparative transcriptome analysis reveals induction of apoptosis in chicken kidney cells associated with the virulence of nephropathogenic infectious bronchitis virus, Microb. Pathog. 113 (2017) 451–459.
[7] R. Chhabra, S.V. Kuchipudi, J. Chantrey, K. Ganapathy, Pathogenicity and tissue tropism of infectious bronchitis virus is associated with elevated apoptosis and innate immune responses, Virolgy 488 (2016) 232–241.
[8] X.L. Feng Cong, Zongni Han, Yuhao Shao, Xiangang Kong, Shengwang Liu*, Transcriptome analysis of chicken kidney tissues following coronavirus avian infectious bronchitis virus infection, BMC Genom. (2013) 14.
[9] D. Bartel, MicroRNA: target recognition and regulatory functions, Cell 136 (2009) 215–233.
P. Bellare, D. Ganem, Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation, Cell Host Microbe 6 (2009) 570–575.

Z. Zhu, Y. Qi, A. Ge, Y. Zhu, K. Xu, H. Ji, et al., Comprehensive characterization of serum microRNA profile in response to the emerging avian influenza A (H7N9) virus infection in humans, Viruses 6 (2014) 1525–1539.

J.M. Luna, T.K. Schreel, T. Danino, K.S. Shaw, A. Mele, J.J. Fak, et al., Hepatitis C virus RNA functionally sequesters miR-122, Cell 160 (2015) 1099–1110.

A. Zhai, J. Qian, W. Kao, A. Li, Y. Li, J. He, et al., Borna disease virus encoded phosphoprotein inhibits host innate immunity by regulating miR-155, Antivir. Res. 98 (2013) 66–75.

J. Bhanja Chowdhury, S. Shrivastava, R. Steele, A.M. Di Bisceglie, R. Ray, R.B. Ray, Hepatitis C virus infection modulates expression of interferon stimulatory gene IFITM1 by upregulating miR-155, Antivir. Res. 98 (2013) 66–75.

W.Q. Fan, H.N. Wang, Y. Zhang, Z.B. Guan, T. Wang, C.W. Xu, et al., Comparative dynamic distribution of avian infectious bronchitis virus M41, H120, and SAIBK strains by quantitative real-time RT-PCR in SPF chickens, Biosci. Biotechnol. Biochem. 76 (2012) 2255–2260.

S. Wu, L. He, Y. Li, T. Wang, L. Feng, L. Jiang, et al., miR-146a facilitates replication of dengue virus by dampening interferon induction by targeting TRAF6, J. Infect. 67 (2013) 329–341.

V. Punj, H. Matta, S. Schamus, A. Tameswitz, B. Anyang, P.M. Chaudhary, Kaposi's sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 suppresses CXCR4 expression by upregulating miR-146a, Oncogene 29 (2010) 1835–1844.

C.R. Stewart, G.A. Marsh, K.A. Jenkins, M.P. Gantier, M.L. Tizard, et al., Promotion of Hendra virus replication by microRNA 146a, J. Virol. 87 (2013) 3782–3791.

M. Muzio, J. Ni, P. Feng, V.M. Dixit, IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling, Science 278 (1997) 1612–1615.

J. Hou, P. Wang, L. Liu, X. Liu, F. Ma, H. An, et al., MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2, J. Immunol. 183 (2009) 2150–2158.