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Short communication

The expression profile of miR-222b-5p/MAPK10 in spleens of SPF chickens infected with REV-SNV at 28-42 dpi

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Abstract

Reticuloendotheliosis virus (REV) is an avian oncogenic retrovirus that causes atrophy of immune organs, such as the spleen, thymus, and bursa of Fabricius, leading to severe immunosuppression. However, there is limited information describing the genes or microRNAs (miRNAs) that play a role in replicating REV-spleen necrosis virus (SNV). Our previous miRNA and RNA sequencing data showed that the expression of gga-miR-222b-5p was significantly upregulated in REV-SNV-infected chicken spleens of 7, 14, and 21 dpi compared to non-infected chicken spleens, but mitogen-activated protein kinase 10 (MAPK10), which is related to innate immunity, had the opposite expression pattern. To understand chicken cellular miRNA function in the virus-host interactions during REV infection, we used quantitative reverse transcription PCR (qRT-PCR) to determine whether the expression of gga-miR-222b-5p and MAPK10 in the spleen of specific-pathogen-free chickens at 28, 35, and 42 dpi was consistent with the first 3 time points, and dual-luciferase reporter assay was used to determine the targeting relationship between gga-miR-222b-5p and MAPK10. Results show that MAPK10 was downregulated at all 3 time points; however, significant difference ($p<0.01$) was noted only at 35 dpi. Moreover, the expression of gga-miR-222b-5p was upregulated; however, significant difference ($p<0.01$) was observed only at 28 and 35 dpi. A dual-luciferase reporter assay showed that MAPK10 is a direct target of gga-miR-222b-5p. This study suggests that gga-miR-222b-5p may target MAPK10 to promote the REV-SNV-induced tumorigenesis via the RLRs signaling pathway.

Key words: reticuloendotheliosis virus strain SNV, RLRs signaling pathway, gga-miR-222b-5p, mitogen-activated protein kinase 10, a dual-luciferase reporter gene experiment

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Introduction

Reticuloendotheliosis virus (REV) is an avian oncogenic retrovirus that causes atrophy of immune organs, such as the spleen, thymus, and bursa of Fabricius, leading to severe immunosuppression. However, there is limited information describing the genes or microRNAs (miRNAs) that play a role in replicating REV-spleen necrosis virus (SNV).

MiRNAs are small endogenous (22-25 nt in length) non-coding RNAs that are ubiquitously expressed in higher eukaryotic cells (Bi et al. 2018). They regulate genes after transcription by controlling the translation of mRNAs into proteins. Previous research revealed that abnormal expression of miRNAs is associated with tumorigenesis (Fiorucci et al. 2015). Studies have shown that after REV infection of chicken embryonic fibroblast (CEF) cells, miR-155 inhibits apoptosis by upregulating expression and accelerating cell life cycle to promote REV infection (Yao et al. 2017, Gao et al. 2020). In addition, gga-miR-221, gga-miR-222 and gga-miR-375 also play a key role in tumorigenesis caused by subgroup J avian leukosis virus (Lee et al. 2014, Dai et al. 2015).

Mitogen-activated protein kinase (MAPK) is a double phosphorylation-activated protein kinase that exists in all eukaryotes. It contains 11 highly conserved subdomains in different species and is the core kinase of the MAPK cascade pathway. As an important signaling molecule downstream of RIG-I-like receptors (RLRs) signaling pathway, MAPK10 plays an important role not only in innate immunity of chickens but also in human oncology and brain neurological diseases, which are more common targets of related miRNAs (Ying et al. 2006, Kunde et al. 2013).

The spleen as the largest lymphoid organ in birds contains a large number of immunocompetent cells, and is also the center of cellular and humoral immunity which plays a very important immunological role in birds. Hence, we chose the spleen as our main research object (Sandford et al. 2011).

Our previous results (Gao et al. 2019a) from artificial infection test of specific-pathogen-free (SPF) chickens at earlier time points of 7, 14 and 21 days after infection with REV-SNV at 1 day of age showed that gga-miR-222b-5p was upregulated and MAPK10 was downregulated in the experimental group of chicken spleen infected with REV-spleen necrosis virus (SNV) compared with the control group, and the expression of both yielded a negative correlation.

Here, we used bioinformatics methods to analyze the direct target genes and action sites of gga-miR-222b-5p aside from using qRT-PCR in detecting the expression of gga-miR-222b-5p and MAPK10 at 28, 35, and 42 dpi. Finally, we used a dual-luciferase reporter gene system to find out whether gga-miR-222b-5p can target and regulate the MAPK10 gene. The work above is helpful to further understand the molecular regulation mechanism of RLRs signaling pathway mediated by miRNA in REV-SNV-induced immunosuppression.

Materials and Methods

Total RNA Isolation

The REV strain SNV (GenBank: DQ003591.1) was kindly provided by Professor Shuhong Sun and Zhizhong Cui from Shandong Agricultural University.

Spleen tissues were collected in our previous SPF chickens experiment (Gao et al. 2019a).

Total RNA was extracted using RNA extraction kit (CWBIO, Beijing, China).

SYBR Green-qRT-PCR for MAPK10 and gga-miR-222b-5p

We employed qRT-PCR to confirm the expression levels of MAPK10 and gga-miR-222b-5p. Primers that were specific to MAPK10 and gga-miR-222b-5p were designed using Premier Primer 5.0 software. All the primers used in this study are listed in Table 1. The kits were the One-Step TB Green® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) and Mir-X™ miRNA First-Strand Synthesis (TaKaRa, Dalian, China). The GAPDH and U6 small nuclear RNA (snRNA) were used as the internal reference genes. The relative expression values were normalized by the internal control. We used LightCycler®96 (Roche Diagnostics GmbH, Germany) for the quantitative real-time PCR analysis, following the manufacturer's instructions. Triplicate qRT-PCR was performed on each RNA sample to ensure the reproducibility of the amplification. After amplification, the relative fold change of the differentially expressed mRNA and miRNA was calculated through the $2^{-\Delta\Delta CT}$ method.

Dual-luciferase reporter assay

We predicted a possible target relationship between MAPK10 and gga-miR-222b-5p by TargetScan and miRBD, and the predicted results showed the presence of three sites paired with gga-miR-222b-5p at 3'UTR of MAPK10.

MAPK10 primers from Premier Primer 5.0 software were designed to amplify the target fragment. Enzymatic cleavage was added at the 5′end of the primer, which were TTGGAGCTC and GACTCTAGA.

Table 1. Primers used in this study.

Primers for qRT-PCR
MAPK10 F ATGCCAAGCGTGCTTACAGAGA
MAPK10 R TGGCATCCATCAGCTCCATC
gga-miR-222b-5p TGCTCAGTAGTCAGTGTAGGATCTGT
Primers for a Dual-luciferase reporter assay
MAPK10 F1 TTGGAGCTCGATGAGGCTGCACAGCTT
MAPK10 R1 GACTCTAGATTGTCACAAGCAAAGGAACG
MAPK10 F2 TTGGAGCTCTTCAAAATGCTGCCACGA
MAPK10 R2 GACTCTAGATATTCTGCATGTTTGAATTCAC

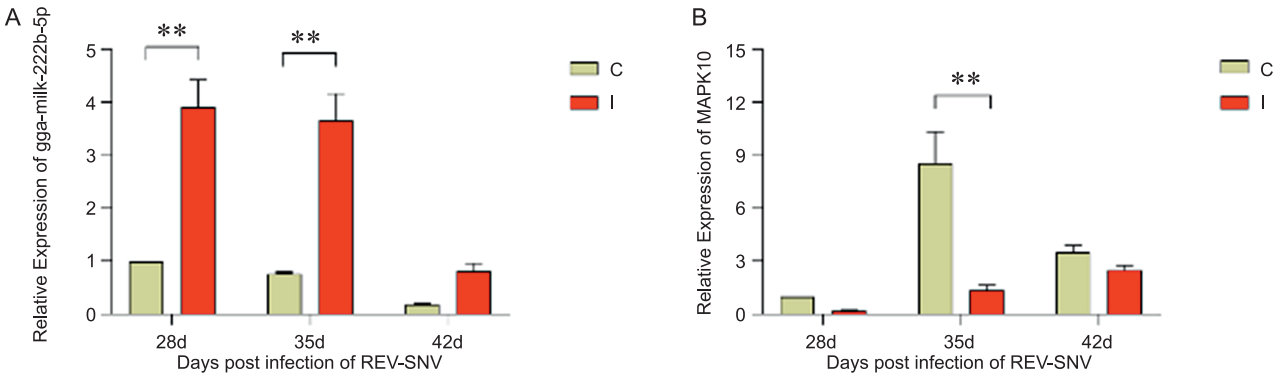

Fig. 1. The differential expression of gga-miR-222b-5p in the spleen of specific-pathogen-free chickens at 28, 35, and 42 dpi between the experimental group and control group, **p<0.01 (A). The differential expression of mitogen-activated protein kinase 10 (MAPK10) in 28, 35, and 42 dpi between the experimental group and the control group, **p<0.01 (B).

Primers and target fragments were synthesized by the external commercial service.

After a recombinant plasmid construction was completed, a double enzyme digestion test was carried out using Thermo Scientific FastDigest XbaI and SacI (Thermo Scientific, USA). The CEF cells (SPF White Leghorn line chickens; Jinan SAIS Poultry Co. Ltd., China) were cultivated adherently in cell culture vessels containing Dulbecco's Modified Eagle Medium (DMEM; Biological Industries, IL, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, IL, USA) at 37°C in an atmosphere of 5% CO_2. CEF cells were seeded at a density of 1×10^5 cells per well in 24-well plates. After 24 h, the cells were co-transfected with 500 ng pmir-GLO WT-MAPK10--3′UTR (wild type) or pmir-GLO-MT-MAPK10-3′UTR (mutant-type) plasmids, then 300 ng gga-miR-222b-5p mimics or miR-NC with the Lipofectamine 2000 Reagent was used. 36 h after transfection, luciferase assays were performed using a luciferase detector (Turner BioSystems, USA). We used Dual-GLO® Luciferase Assay System Kits (Promega, Madison, WI, USA) to measure luciferase activity, following the manufacturer's instructions.

Results and Discussion

The present results show that MAPK10 was downregulated at all 3 time points; however, a significant difference ($p<0.01$) was noted only at 35 dpi (Fig. 1B). Moreover, the expression of gga-miR-222b-5p was upregulated; however, a significant difference ($p<0.01$) was observed only at 28 and 35 dpi (Fig. 1A). We used the miRNA target prediction software miRDB. MAPK10 was predicted to be a potential target gene of gga-miR-222b-5p. Consequently, the mature sequence of gga-miR-222b-5p and the binding seed sequence of MAPK10 were obtained (Fig. 2A). We constructed wild-type and mutant-type plasmids and verified by double enzyme digestion (Fig. 3). A dual-luciferase reporter assay showed that MAPK10 is a direct target of gga-miR-222b-5p (Fig. 2B).

MiR-222-5p is a member of the miR-222 family, which is highly conserved on the X chromosome in humans, rats, and mice (Garofalo et al. 2012). In chickens, miR-222 is located on chromosome 1 and its expression is not only upregulated in avian oncology (Li et al. 2012), but also has been widely reported in many human oncologic diseases (Chun-zhi et al. 2010). MiR-222-5p was upregulated in chicken bursa of Fabricius (Yu et al. 2017) as same as our previous results in the spleen of chickens infected with REV-SNV (Gao et al. 2019a).

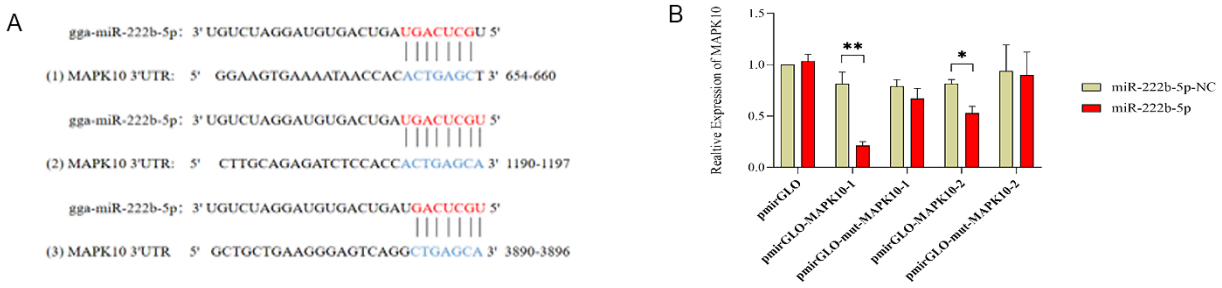

Fig. 2. The 3'UTR target sequence of gga-miR-222b-5p in MAPK10 (A). Experimental results of dual-luciferase reporter gene of gga-miR-222b-5p. 1 represents a recombinant plasmid containing the first two segments of the target; 2 represents a recombinant plasmid containing the last target segment, **p<0.01; *p<0.05 (B).

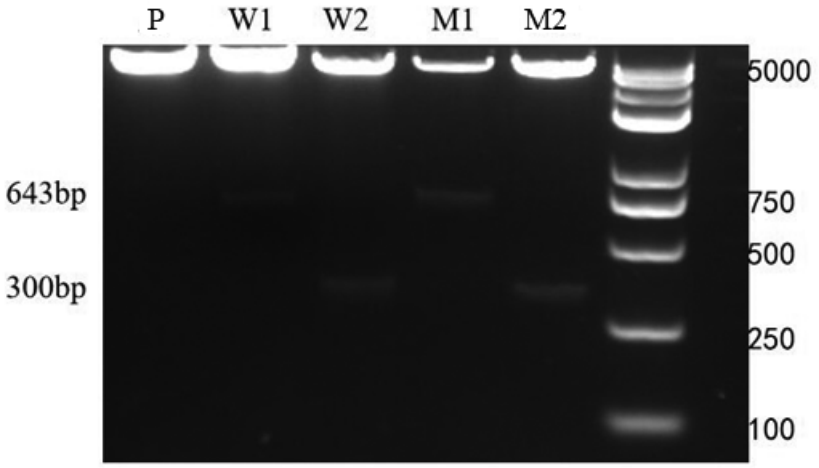

Fig. 3. The results of double enzyme digestion. P refers to the pmir-GLO empty plasmid; W1 refers to the pmir-GLO wild-type recombinant plasmid containing the first two targets; W2 refers to the pmir-GLO wild-type recombinant plasmid containing the last target; M1 refers to the pmir-GLO mutant recombinant plasmid containing the first two targets; M2 refers to the pmir-GLO mutant recombinant plasmid containing the last target.

Finally, we wondered whether gga-miR-222b-5p inhibited the apoptosis of cancer cells by targeting MAPK10, via the RLRs signaling pathway, which enhanced the pathogenicity of REV-SNV. Are there relevant transcription factor binding sites for Rel? Since the time points are a little bit long after infection, this may also be an indirect effect of tumorigenesis rather than a direct effect of REV. All of the above questions will need to be clarified in the further experiments.

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