# MiR-20a-5p is Multifunctional Regulator in Chickens Immune Responses Induced by NDV, IBDV and AIV Vaccines Respectively

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**Short Report**

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# Abstract

MiR-20a is an important regulator of immune function. To further explore the expression and functional characteristics of miR-20a in different immune responses, the spatiotemporal expression characteristics of miR-20a-5p in the immunized chicken models induced by three poultry vaccines (infectious bursal disease attenuated vaccine, Newcastle disease attenuated vaccine and avian influenza inactivated vaccine) were analyzed. The results showed that the expression levels of serum circulating miR-20a-5p were significantly different in different stage of the three immune responses. The expression patterns of tissue miR-20a-5p at two time points (5dpi and 21dpi) were similar between ND group and H9 group, but different from IBD group, and the spleen, thymus and bursa of *Fabricius* were the possible key tissues for miR-20a-5p playing immune regulatory functions in different immune responses. This study showed that miR-20a-5p was a multifunctional key factor involved in different immune responses, and provided a reference for further exploring the immune regulation function and potential application of miR-20a.


# Introduction

MiR-20a, a member of miR-17-92 family, has an important regulatory effect on a variety of immunocytes. For instance, miR-20a can promote the differentiation of follicular helper T cells (Tfh), and inhibit the TCR-mediated signaling and cytokine production in human naïve $CD4^+$ T cells (Baumjohann et al. 2013; Reddycherla et al. 2015). Moreover, miR-20a can regulate the response and immunoglobulin production of B cells (Ventura et al. 2008; Xu et al. 2015), enhance the infiltration, phagocytosis and pro-inflammatory cytokines secretion of macrophages (Zhu et al. 2013), and decrease the cytotoxicity and sensitivity of NK cells (Xie et al. 2014; Zhu et al. 2018; Tang et al. 2019). In addition, miR-20a inhibits autophagy process by regulating multiple targets and multiple pathways, which can defense against invading pathogens in innate immune response (Guo et al. 2016). So miR-20a participates in the immune functions of different immune-related cells and has become one of the hotspots in the study of immune function regulation. However, the expression characteristics and functions of miR-20a between different immune responses have not been reported.

In this study, three classical epidemic vaccines (infectious bursal disease attenuated vaccine, Newcastle disease attenuated vaccine and avian influenza inactivated vaccine) were used to prepare immune response chicken models, then the expression characteristics and change rules of serum circulating and immune-related tissue miR-20a-5p at different stages of the three vaccines immune response processes were analyzed. The study can provide a reference for in-depth exploration of the immune regulation function and application of miR-20a.

# Materials And Methods

### 1.1 Ethics Statement

This animal study protocol was approved by the Animal Care and Use Institutional Committee (IACUC) of Harbin Normal University (approval number: No. SYXKHEI2008006). Chickens were raised and managed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China. All efforts were made to comply with animal welfare guidelines and minimize animal suffering.

### 1.2 Experimental grouping and sample collection

Two hundred one-day-old healthy chickens were purchased from Xiangfang farm in Harbin and equally divided into control group, IBD group, ND group and H9 group. Chickens were free to feed and drink. At 12-day-old, the chickens of ND group and IBD group were respectively administered with Newcastle disease virus (NDV) LaSota attenuated vaccine (Harbin veterinary research institute, China) and B87 subtype infectious bursal disease virus (IBDV) attenuated vaccine (Harbin veterinary research institute, China) by eye drop, and the chickens of H9 group were injected into cervical subcutaneous with H9 subtype avian influenza virus inactivated vaccine (Harbin veterinary research institute, China). The chickens of control group were administered with vaccine dilute solution (Harbin veterinary research institute, China) by eye drop. Three chickens of each group were randomly selected and the heart, thymus, spleen, bursa of *Fabricius*, small intestine, cecal tonsil, colon, glandular stomach and serum were collected on 1, 2, 3, 4, 5, 7, 14, 21, 28 and 35 day post-immunization (dpi). All samples were frozen in liquid nitrogen and stored at -80℃.

### 1.3 Antibody detection

The IBDV serum antibody concentrations of IBD group were detected with an ELISA kit (Ruixin Biotechnology Company, China) according to the instructions, the NDV and AIV serum antibody levels were detected with hemagglutination and hemagglutination inhibition (HA-HI) experiments using the NDV and AIV standard antigen from Harbin Veterinary Research Institute, China.

### 1.4 Quantitative real-time PCR (qRT-PCR)

The reverse transcription reaction of miR-20a-5p was performed with FSQ-301 kit (TOYOBO, Shanghai) according to the instructions. The reverse transcription primer of miR-20a-5p was 5´-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACCTGC-3´, and the reverse transcription primer of internal reference *U6* was 5 ´-AACGCTTCACGAA TTTGCGT-3 ´. QRT-PCR was used to analyze the expression levels of miR-20a-5p from the different groups, and the miR-20a-5p primers were 5´-ACACTCCAGCTGGGTAAAGTGCTTATAGTGC-3´ and 5´-ACACTCCAGCTGGGTAAAGTGCTTATAGTGC-3´. The qRT-PCR system was 10 μL, including 5 μL SYBR (TOYOBO, Shanghai), 0.2 μL ROX (TOYOBO, Shanghai), 0.3 μL each primer, 1 μL cDNA (100 ng), 3.5 μL dd$H_2O$. The reaction procedures were 95°C/1 min; 45 cycles of 95°C/15s, 60°C/30s, 72°C/30s. The qRT-PCR system and reaction procedures of *U6* were the same as that of miR-20a-5p, and the primers of *U6* were 5´-CTCGCTTCGGCAGCACA-3´ and 5´-AACGCTTCACGAATTTGCGT-3´.

### 1.5 Statistical analysis

All experimental techniques were repeated three times, and the expression levels of miR-20a-5p were calculated by the $2^{-\triangle ct}$ method. The data were analyzed using SPSS 20.0 software and graphed with GraphPad Prism 8.0.

# Results And Discussion

Newcastle disease, avian influenza and infectious bursal disease are the three most common infectious diseases in the poultry production, which have brought huge losses to the poultry industry. In this study, the classical vaccines of the three diseases were used to prepare chicken immune models, so the research results not only provide references for in-depth understanding the functions and characteristics of miR-20a-5p in different vaccine immune responses, but also have positive research significance for the immune prevention and treatment of the three diseases. Serum antibody detection results showed that the antibody levels of the IBD group, ND group, and H9 group gradually increased after immunization, reaching a peak on 21 dpi and then slowly decreasing, while no antibody was detected in the control group, indicating that the three vaccine-immunized chicken models were successfully prepared (the results were in attachment 1) (Wang 2020; Liu 2020).

### 2.1 Expression characteristics analysis of serum circulating miR-20a-5p in different immune responses

Analyzing the changes in the expression levels of serum circulating miR-20a-5p can indicate the functional characteristics of miR-20a-5p in different immune responses. The qRT-PCR results showed that, in IBD group, except for the significantly up-regulation on 21dpi ($P$<0.01), circulating miR-20a-5p were all down-regulated at the other time points and especially during 1-5 dpi ($P$<0.05). In ND group, the serum levels of miR-20a-5p were significantly up-regulated on 2dpi and 3dpi during the innate immunity stage (1-4dpi) (Abbas 2012; Murphy 2011) ($P$0.05); during the transition stage from innate immunity to acquired immunity, miR-20a-5p was significantly up-regulated on 5dpi ($P$0.05); during the acquired immunity period (7-35dpi) (Abbas 2012; Murphy 2011), circulating miR-20a-5p was significantly down-regulated on 7dpi and 28dpi, but significantly up-regulated to the peak ($P$<0.01) on 21dpi. In H9 group, the circulating miR-20a-5p was down-regulated at all time points and especially on 2dpi, 21dpi and 28dpi ($P$<0.01) (Figure 1).

By comparison with the control group, circulating miR-20a-5p showed a similar downregulation change pattern in IBD group and H9 group from 1dpi to 5dpi, which was the opposite to ND group. The results suggested that circulating miR-20a-5p actively participated in the innate immune response and the initial response of acquired immunity of the three immune responses, but there were differences in functions. Whether the significant down-regulations of miR-20a-5p in IBD group and H9 group during the innate immunity stage were related to the immunosuppression of IBDV (Gimeno and Schat 2018) and inactivated H9 vaccine remained to study in depth. Interestingly, in the acquired immunity stage, circulating miR-20a-5p showed the most significant differences and changes on 21dpi (significant

increasing in IBD and ND groups, but decreasing in H9 group), which indicated that 21dpi was a key time node worth exploring.

### 2.3 Expression characteristics analysis of tissue miR-20a-5p in different immune responses

Through comprehensively analyzing the changes of serum circulating miR-20a-5p in different immune responses, we selected the three time points of 2dpi (innate immunity stage), 5dpi (transition stage from innate immunity to acquired immunity) and 21dpi (acquired immunity stage) with significant differences to further compare and analyze the expression characteristics of tissues miR-20a-5p in the three immune responses. On 2dpi, compared with the control group, miR-20a-5p was significantly up-regulated in the IBD group ($P$<0.01); in the ND group, except for the down-regulation of thymus, cecal tonsil and glandular stomach, miR-20a-5p was significantly up-regulated in the other tissues ($P$<0.01); in the H9 group, except for the thymus and spleen, miR-20a-5p was significantly up-regulated in the other tissues ($P$<0.01) (Figure 2A). These results suggested that the roles of miR-20a-5p in the innate immunity of these three vaccines were different, but there were certain similarities between IBD group and H9 group according relative expression levels, and different from ND group, which was consistent with the results of serum analysis.

On 5dpi, compared with the control group, miR-20a-5p was significantly down-regulated in the spleen, thymus, and glandular stomach in the IBD group ($P$<0.05), and up-regulated in the other tissues; in the ND group, miR-20a-5p was significantly down-regulated in all candidate tissues except for the small intestine ($P$<0.05); in the H9 group, miR-20a-5p was up-regulated in the cecal tonsil and glandular stomach, but significantly down-regulated in the other tissues (except for colon) ($P$<0.01) (Figure 2B). The results showed that miR-20a-5p actively participated in the initial stage of the acquired immunity stage. On the whole, the expression patterns of the ND group and H9 group were similar, and which had a great difference from that of IBD group.

On 21dpi, compared with the control group, miR-20a-5p was significantly up-regulated in the heart, colon, and cecal tonsils in the IBD group ($P$<0.05) and significantly down-regulated in the spleen, small intestine, and glandular stomach ($P$<0.05); in the ND group, miR-20a-5p was significantly down-regulated ($P$<0.01), but significantly up-regulated in the bursa and no change in the heart; in the H9 group, except for the up-regulation of miR-20a-5p in the bursa, it was down-regulated in the other tissues (Figure 2C). The results showed that miR-20a-5p actively participated in the phase of acquired immunity. Basically, the expression patterns of the ND group and H9 group were also similar, and which were different from the IBD group.

By comparing the changes of expression levels between the three time points, in control group, we interestingly found that miR-20a-5p showed a significant up-regulation trend in the spleen from 2dpi to 21dpi. Studies showed that miR-20a could promote Th cell differentiation (Wu et al. 2018), indicating that miR-20a-5p was positively related to the development of chicken spleen. In the IBD group, the expression levels of miR-20a-5p in the bursa of *Fabricius* showed a significant downward trend from 2dpi to 21dpi, and miR-20a was involved in regulating B cell responses (Xu et al. 2015; Wu et al. 2018), suggesting that miR-20a-5p was negatively related to the immune response in bursa of *Fabricius*. In addition, the expression levels of miR-20a-5p had similarities and differences in different immune treatment groups.

For example, miR-20a-5p was significantly up-regulated in the heart, bursa of *Fabricius*, colon and small intestine in the three groups on 2dpi, significantly down-regulated in the spleen and thymus on 5dpi, and significantly down-regulated in the spleen and glandular stomach on 21dpi, suggesting that miR-20a-5p performed similar functions in different stages of the three immune responses. However, the differences of miR-20a-5p in the three immune responses were more significant than its similarities. For instances, in IBD group, miR-20a-5p was up-regulated in the thymus on 2dpi, colon on 5dpi, and cecal tonsil on 21dpi, while it was down-regulated in the same tissues in ND group and H9 group; and in IBD group, miR-20a-5p was significantly up-regulated in the heart on 21dpi, but no significant changes in ND group and H9 group. Basically, the expression distributions of tissue miR-20a-5p of ND group and H9 group at the last two time points were similar, which were different from that of IBD group, indicating that miR-20a-5p expression was related to the type of vaccine and tropism of virus, but the specific mechanism needed to be studied in the future.

In conclusion, miR-20a-5p actively participated in the whole processes of immune responses and played different roles in different immune responses. 2dpi, 5dpi and 21dpi were the possible key time points for the significant changes of serum circulating miR-20a-5p in the three immune responses, and the spleen, thymus and bursa of *Fabricius* were the differentially expressed tissues worthy of attention. This study can provide a positive theoretical reference for further studying the immune function of miR-20a-5p and its potential application as a molecular marker.

# Declarations

### -Consent to participate

Yiru Wu: main performed the experiments, analyzed the data and wrote the paper.

Yang Liu, Qiuyuan Wang, Jie Wen, Jianwei Han and Yufei Tian: contributed help and participated in operation of the study.

Chaolai Man: conceived and designed the present study, and revised the paper.

### -Consent for publication

The authors declare that there are no competing interests associated with the manuscript. This work was supported by the Science Foundation of Heilongjiang Province (LH2019C073) and Postgraduate Innovation Project of Harbin Normal University (HSDSSCX2021-06).

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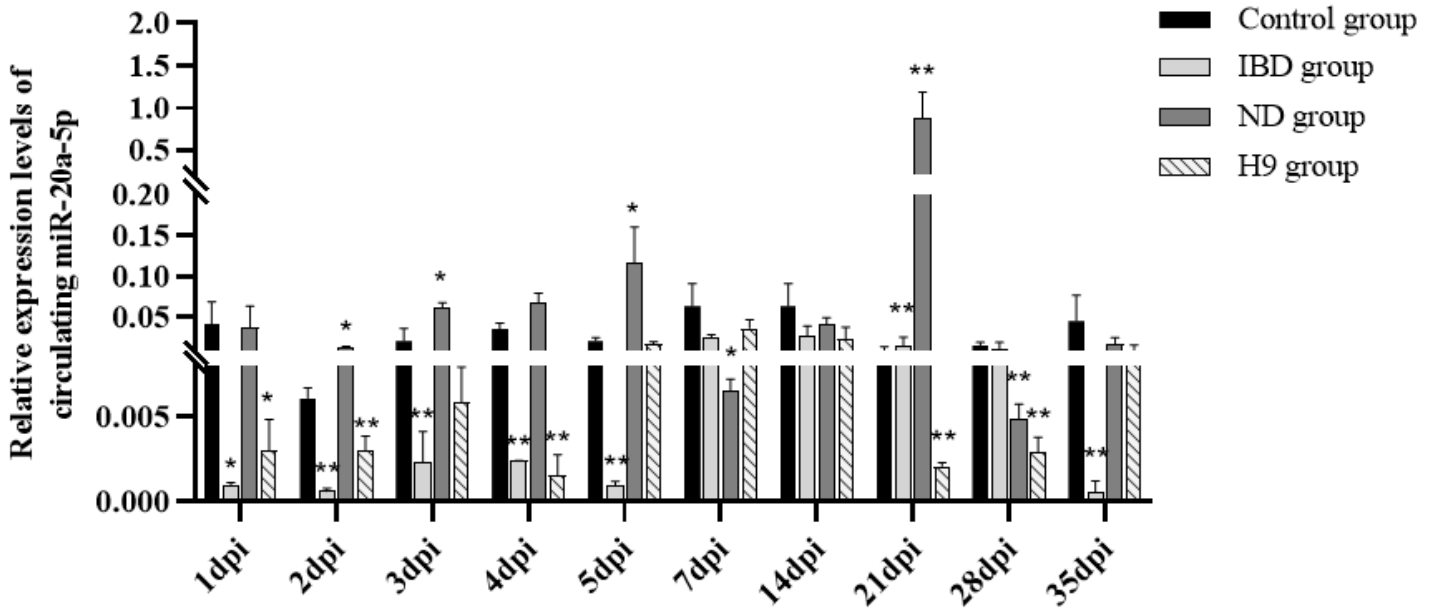
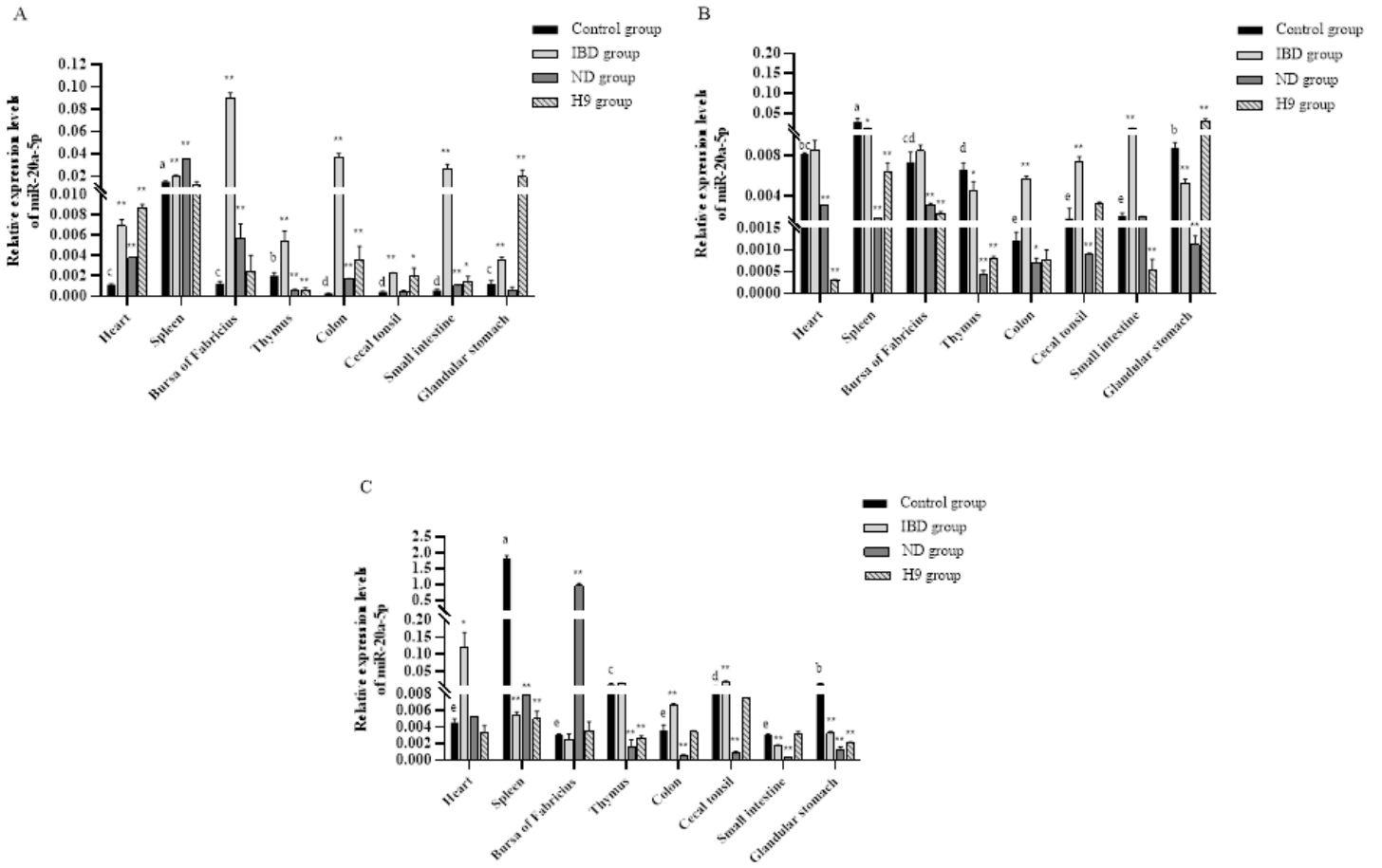
# Figures




## Figure 1

Expression levels of serum circulating miR-20a-5p in different immune responses The abscissa was the number of day post-immunization (dpi). * indicated statistically significant (P<0.05) on the same day between control group and vaccine group, and ** indicated statistically highly significant (P<0.01) on the same day between control group and vaccine group.




## Figure 2

The expression characteristics of tissues miR-20a-5p in different vaccine immune responses A, B, and C were the expression levels results of tissues miR-20a-5p in different immune responses on 2dpi, 5dpi and 21dpi, respectively. The abscissa represented different tissues, and the ordinate represented relative expression levels. * indicated statistically significant (P<0.05) in the same tissue between control group and vaccine group, and ** indicated statistically highly significant (P<0.01) in the same tissue between control group and vaccine group. Different letters indicated that different tissues of control group had significant differences at the same time point (P<0.05), while the same letters indicated that different tissues of control group had no significant differences at the same time point (P>0.05).