

# Research Note: IsomiRs of chicken miR-146b-5p are activated upon *Salmonella enterica* serovar Enteritidis infection

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**ABSTRACT** In order to enrich the knowledge of chicken transcriptomic response to *Salmonella enterica* serovar Enteritidis infection, 2-day-old chicks were orally inoculated with this bacteria ( $1.0 \times 10^8$  cfu/mL), and then the cecum tissues of 3 days post-inoculation were utilized for RNA sequencing (6 replicates each for treatment group and control group). After analysis, we found a variety of inflammatory genes were triggered at the mRNA level upon infection. Notably, the expression profiles at the miRNA level and the isomiR level were

heterogeneous. Certain isomiRs of chicken miR-146b-5p were significantly increased by more than 2 times compared to control ( $P_{adj} < 0.05$ ). Combining the bioinformatics prediction, transcriptome data and RT-qPCR results, we deduced that the isomiRs of chicken miR-146b-5p might act to sustain the RIG-I-like receptor signaling and type I interferon induction by repressing *USP3* transcript. Our findings provide a new perspective on the regulatory function of miR-146b-5p and facilitate the study of isomiRs.

**Key words:** chicken, miR-146b-5p, isomiR, RIG-I-like receptor, interferon

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## INTRODUCTION

*Salmonella enterica* serovar Enteritidis (**S. Enteritidis**) is a non-host specific, zoonotic pathogen, which appears in the most common clinical isolates of both human and poultry. Avian innate immunity is considered similar to the mammalian system in terms of structure and function. Characterizing the avian immunity genes would allow targeting of crucial regulators in both avian and mammal infections (Beacon and Davie, 2021).

It is known that miR-146b-5p contributes to the immune homeostasis and mainly plays a negative role in the activation of innate immune response. We observed that some key target genes validated in humans, such as *IRAK1*, *TRAF6* (Park et al., 2015), *IFI35* (Zhang et al., 2021a), and *TLR4* (Ouyang et al., 2021) lack their corresponding target sites or 3'-UTRs directly in chickens, indicating that the targeting effect of these genes is weakened and the regulatory function of miR-146b-5p may be realized in other ways.

Most miRNA studies focused on the canonical ones, which sequences are annotated in miRBase database. Recently, more and more deep sequencing data and experiments supported the existence and function of isomiRs, the sequence variants of annotated miRNA, hinting the importance of including them in miRNA study. Herein, we designed an experiment on *S. Enteritidis* infection of chickens and considered isomiRs in the transcriptome analysis for a higher resolution in small RNA data.

## MATERIALS AND METHODS

### Animals and Experimental Design

The chicks used in this experiment were 2 populations of reciprocal crosses, which derived from 2 Chinese local chickens, Guangxi Yao and Jining Bairy. The cross (Guangxi Yao ♂ × Jining Bairy ♀) and the reverse-cross (Guangxi Yao ♀ × Jining Bairy ♂) were provided by Shandong Bairy Chicken Breeding Co., Ltd (Jining, China) separately. Thus the experiment was carried out in 2 batches. To each crossbreed, 100 mixed gender, *Salmonella*-negative chicks were randomly divided into 2 groups at after hatching. They were housed in different chambers (32–35°C, 50 to 60% RH, and 24 h light) with ad libitum access to water and commercial feed without antibiotic treatment. The strain of *S. Enteritidis*

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(CVCC3377) was purchased from China Veterinary Culture Collection Center (Beijing, China) and stored at  $-80^{\circ}\text{C}$ . After rejuvenation with nutrient broth (Hopebio, Qingdao, China), the bacterial solution was centrifuged at 4,000 rpm for 5 min and suspended again with sterilized PBS to OD value 1 (about  $1.0 \times 10^8$  cfu/mL). At 2-day old, the chicks of one group were orally inoculated with 0.3 mL *S. Enteritidis* as treatment group, and the chicks of the other group were mock inoculated with the same volume of PBS as control group. At 3 days postinoculation (**dpi**), partial of the chicks were sacrificed. The cecum tissues were taken out, quick-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA sequencing. One chick was a replicate and there were 3 replicates in each group. The remanent challenged chicks had no obvious infection symptoms until 14 dpi. The experimental procedure was repeated on another crossbreed. Thus 4 groups were generated as cross control (**CC**), cross treatment (**CT**), reverse-cross control (**RC**), and reverse-cross treatment (**RT**). All animal experiments were approved by the Laboratory Animal Management and Use Committee of Shandong Agricultural University (Permit Number: SDAUA-2018-058). We strive to reduce the suffering of animals.

We merged the controls and the treatments into merged control (**MC**) and merged treatment (**MT**), respectively. The theoretical basis of this method is that the reciprocal crosses have similar genetic resistance to pathogen. Another advantage is that it can resist the batch effect (those genes with great variation between 2 controls or 2 treatments will be excluded by the probability test). The false positive rate would be reduced as low as possible and the result should be common across crossbreeds and batches. However, the most unfavorable aspect of this scheme is that it can cover up the genuine differences between 2 crossbreeds, though they were very few in theory. It is hardly avoidable at present. Based on the above reasons, we adopted the 6 vs. 6 analysis scheme.

### Total RNA Isolation, Library Preparation, and Sequencing

Total RNA was extracted from the cecum tissues with TRIzol reagent (Vazyme R401-01). All the RNA samples were high quality (OD260/280, 2.1 to 2.2; OD260/230, 1.8 to 2.2; 28S:18S, 1.9 to 2.3; RIN  $\geq 9.5$ ) verified by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA).

The libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina, Cat.No. RS-122-2301, San Diego, CA) according to the manufacturer's instructions and then were sequenced on an Illumina HiSeq X Ten platform. Average 98.57 M clean reads of each sample were retained, which were mapped to the chicken genome (GRCg7b) using HISAT2. FPKM of each gene was calculated using Cufflinks, and the read counts of each gene were obtained by HTSeq-count. Overall, Q30 base of RNA sequencing was 92.23

to 94.02%, GC content was 45.63 to 47.48%, and the comparison rates between samples and genome was 93.51 to 94.45%.

Total RNA with a mass of 1  $\mu\text{g}$  of each sample was used for the small RNA library construction using TruSeq Small RNA Sample Prep Kits (Illumina). Briefly, total RNA were ligated to adapters at each end. Then the adapter-ligated RNA were reverse transcribed to cDNA and performed PCR amplification. The PCR products ranging from 140 to 160 bp were isolated and purified as small RNA libraries. The libraries were finally sequenced using the Illumina HiSeq X Ten platform. The reads shorter than 15 nt or longer than 41 nt from the raw data were filtered, and the clean reads were obtained. The known miRNAs were identified by aligning against miRBase v22 database, and the unannotated reads were analyzed by mirdeep2 to predict novel miRNAs.

### Identification and Classification of IsomiRs

The tool of IsomiR Seed Extension Aligner was applied to identify, classify, and quantify isomiRs. Clean reads from small RNA-seq were mapped on the known miRNAs sequences using a specialized alignment algorithm developed on top of biological evidence concerning miRNAs structure. A total of 293,833 isomiR sequences were identified, of which 3,151 sequences have an average expression of more than 5 TPM across the 12 samples and accounted for 98.0% of the total expression. Thus they were used for the next analysis.

The sequencing and bioinformatic analysis were performed by OE Biotech Co., Ltd. (Shanghai, China) and on the OECloud (<https://cloud.oebiotech.cn>). The transcriptome data (excluding small RNA) and the small RNA data are deposited in National Genomics Data Center (<https://ngdc.cncb.ac.cn/>) under BioProject accession number CRA005292 and CRA005293, respectively.

### Validation of Omics Data

To validate the RNA-seq results, the reverse transcription-quantitative PCR (**RT-qPCR**) was used to measure gene expression in the same 12 RNA samples as used in RNA-seq study. According to RNA-seq data, we selected 27 of 30 genes that were both the top 100 highest on basemean and the top 100 lowest on Padj value, added 2 genes (*USP3* and *MDA5*), and finally got a total of 29 targets to be validated. *GAPDH* and *ACTB* were selected as the internal controls for genes, and miR-101-3p and *USB1* (also known as *U6*) for miRNA. The primers of genes were designed by the Primer-Blast tool in NCBI. The 5' primers of miRNAs were modified based on their sequences, and the 3' primer of miRNAs was universal presented in the kit. After testing, the amplification efficiency of all primers was near 2, so we can use the comparative CT method ( $2^{-\Delta\Delta\text{Ct}}$ ) to calculate the relative expression level. By comparison, we found that *ACTB* and *GAPDH* were almost equivalent as a

reference, so we only present the qPCR results adjusted by *ACTB*. For the 2 RT-qPCR experiments of genes and miRNAs, RNA samples were reverse transcribed with different kits (Vazyme R223 [Jiangsu, China] for genes; Monad MR05301 [Jiangsu, China] for miRNAs), and quantified with the same kit (Vazyme Q311) and instrument (ABI QuartStudio 5, Singapore). All samples were run in triplicate.

## Statistical Analysis

The test values including *P* value, false discovery rate-adjusted *P* values (*P*<sub>adj</sub>), foldchange (FC), and base-mean were calculated by DESeq2 software. Correlation analysis, 2-tail *t* test and SD value were performed in Microsoft excel 2016.

## RESULTS AND DISCUSSION

### Triggered Inflammatory Response after *S. Enteritidis* Challenge

The 16S rRNA gene sequencing showed that *Salmonella* were detected in treatments (CT and RT) but not in controls (CC and RC), suggesting the success of our challenge work. There were total 15,205 genes in RNA-seq dataset. Compared to MC, there were 525 differentially expressed genes using Deseq2 software with the threshold of  $|\log_2FC| > 1$  and *P*<sub>adj</sub> < 0.05, of which 394 and 131 were up- and down-regulated, respectively. The validation of 29 genes showed that the average correlation coefficient (*r*) between RNA-seq data and qPCR results in 12 samples was  $93.73 \pm 2.96\%$  (mean  $\pm$  SD; Figure 1), which strongly supported the RNA-seq data and the related analyses.

It is well established that in chickens, the immune response to non-host specific *S. Enteritidis* infection switches from earlier proinflammatory to later anti-inflammatory tolerance within a few days, resulting in a persistent cecal colonization. Gene ontology enrichment analysis with the 525 differentially expressed genes showed that all the 30 genes enriched in inflammatory response term were up-regulated in treatment group, supporting the fact that the inflammatory reactions were triggered. Notably, *ACOD1* (FC = 17.49, *P*<sub>adj</sub> = 1.67E-07) and *TNIP3* (FC = 15.78, *P*<sub>adj</sub> = 2.99E-09) were the 2 genes with the highest foldchange in this term. In humans, both *ACOD1* and *TNIP3* are involved in the negative regulation of NF- $\kappa$ B signaling (Gene annotation, NCBI). Consistently, the anti-inflammatory cytokine *IL-10* was increased by 9.42 times (*P*<sub>adj</sub> = 0.015). The sharp rise of *ACOD1*, *TNIP3*, and *IL-10* indicated that the innate immune response was transforming to the anti-inflammatory tolerance at 3 dpi.

In addition, according to a commercial PCR Array of chicken NF- $\kappa$ B signaling pathway (Qiagen [Hilden, Germany], Cat.No. 330231), 81 of 84 genes listed were present in our dataset and 31 of 32 genes were upregulated

significantly compared to control (*P*<sub>adj</sub> < 0.05), suggesting the NF- $\kappa$ B signaling pathway was activated.

### More IsomiRs Than Annotated miRNAs in Response to *S. Enteritidis* Infection

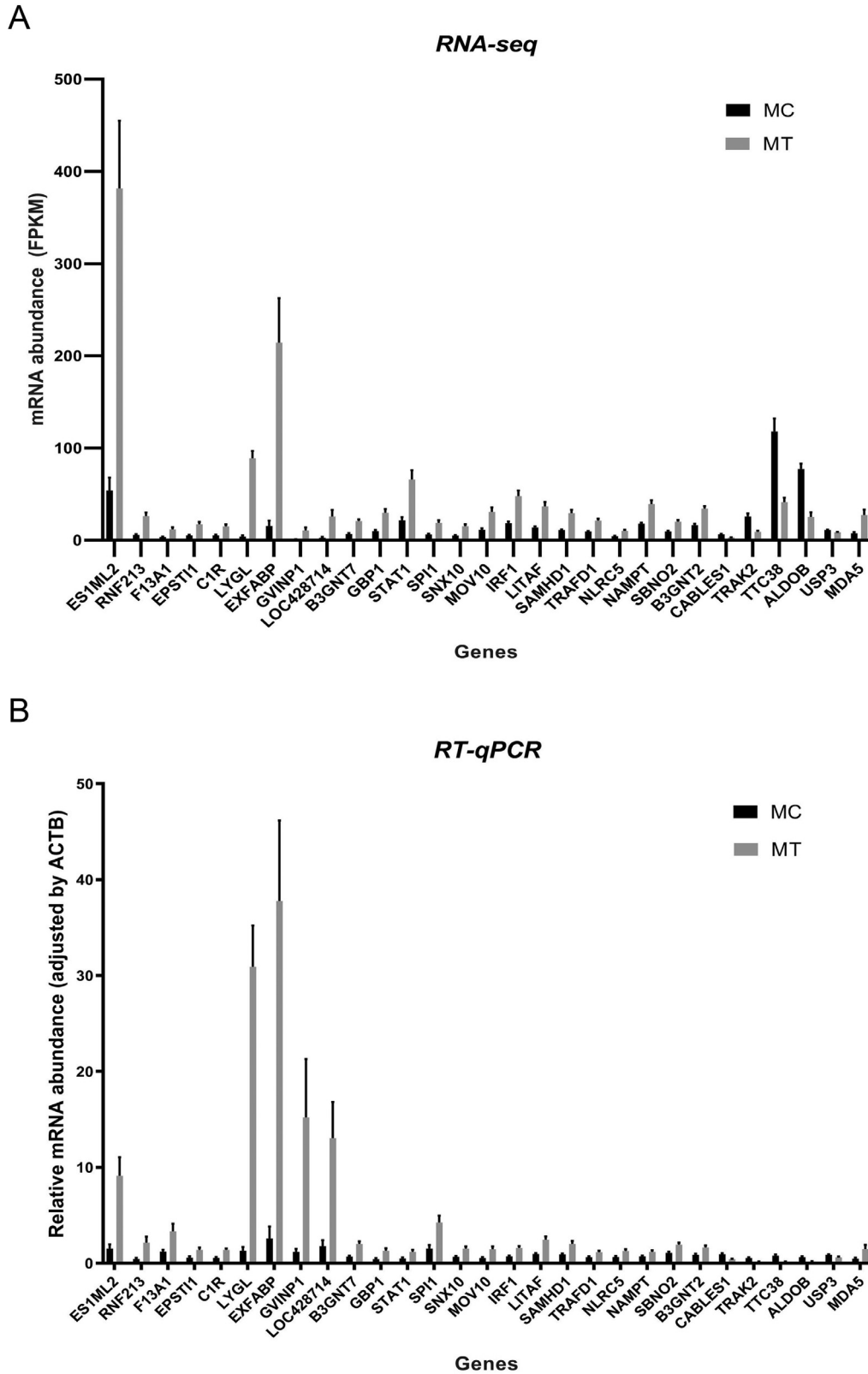
Applying normal miRNA analysis and isomiR-SEA approach, we got 2 datasets from the small RNA-seq data, which included 1,508 annotated miRNA sequences and 3,151 isomiR sequences (TPM >5), respectively. According to the threshold of mRNA analysis, five miRNAs and 36 isomiRs were screened out, respectively. Intriguingly, miR-146b-5p, which was not screened out in normal miRNA analysis (FC = 1.61, *P*<sub>adj</sub> = 0.108), had seven isomiRs and occupied the dominant positions among the top 10 differentially expressed isomiRs in expression (Table 1). The qPCR result validated that miR-146b-5p was increased by 1.98 times (miR-101-3p as internal control, *r* = 0.948) and 2.49 times (*USB1* as internal control, *r* = 0.871). It should be pointed out that the small RNAs were reverse transcribed with tailing reaction, which was insensitive to 3'-terminal variation. The quantitative result should reflect the additive effect of 3'-terminal isomiRs of miR-146b-5p. These data evidenced that miR-146b-5p was increased at isomiR level at 3 dpi.

### Potential Regulation of miR-146b-5p on RIG-I-Like Receptor Signaling

The chicken miR-146-5p family consists of 3 miRNAs, miR-146a-5p, miR-146b-5p, and miR-146c-5p. Their sequences are nearly identical and differ by only 1 to 2 bases at the 3' end. TargetScan tool cannot distinguish these sequences and gives the consensus target genes, 57 predicted with at least one conserved site. Among them, 55 genes were in our dataset and 8 were differential (*P*<sub>adj</sub> < 0.05), including the increased *SPI1*, *DPP9* and the decreased *GRK5*, *RUNX1T1*, *PQLC1*, *ATG7*, *USP3*, *LRIG2* compared to control.

By searching literature, we found that only *SPI1* (also known as *PU.1*) and *USP3* were directly related to miR-146-5p family. *SPI1* is targeted by miR-146-5p in a conserved manner (14 of 18 representative species in TargetScan) and was validated in humans (Nakasa et al., 2011). Interestingly, as a transcription factor, *SPI1* can positively regulate miR-146a-5p expression in humans and mice (Jurkin et al., 2010; Ghani et al., 2011). In light of the significant up-regulation of *SPI1* (FC = 3.02, *P*<sub>adj</sub> = 1.82E-07) after infection, which was validated by qPCR result (2.75 times, *r* = 0.916), we assumed that the chicken miR-146b-5p, which was activated by NF- $\kappa$ B or other signals, could repress the overexpression of *SPI1* protein, and then decrease the transcription of another regulator miR-146a-5p at 3 dpi.

Meanwhile, *USP3* is a member of the ubiquitin specific protease family. The complementary site between *USP3* transcript and miR-146-5p is highly conserved (16 of 18



**Figure 1.** The validation of 29 genes between RNA-seq data (A) and RT-qPCR results (B). The average correlation coefficient ( $r$ ) between them in 12 samples is  $93.73 \pm 2.96\%$  (mean  $\pm$  SD). The bar charts were generated by GraphPad Prism 8.0 software with mean  $\pm$  SEM.

representative species in TargetScan) and was validated in humans (Zhang et al., 2021b). USP3 can dampen type I interferon production by removing K63-linked polyubiquitin chains of RIG-I because the activation of RIG-I signaling requires both RNA binding and the polyubiquitin chains (Cui et al., 2014). Chicken expresses

2 members of the RIG-I-like receptor (RLR) family, MDA5 and LGP2 (Beacon and Davie, 2021). MDA5 shares the same domain architecture with RIG-I (Rehwinkel and Gack, 2020). We speculated that USP3 takes the same action on MDA5 in chickens. Given the increase of miR-146b-5p and the decrease of *USP3*

**Table 1.** The top 10 differentially expressed isomiRs.

isomiR sequence	miRNA family	FC	<i>P</i> <sub>adj</sub>	Mean TPM	
				MC	MT
UGAGAACUGAAUCCAUAAGGCGUU	gga-miR-146b-5p	2.04	1.29E-04	2,732.9	5,622.7
UGAGAACUGAAUCCAUAAGGCGUC	gga-miR-146b-5p	2.09	1.11E-04	396.0	832.4
UGAGAACUGAAUCCAUAAGGCGUUU	gga-miR-146b-5p	2.26	1.99E-05	255.6	580.6
UGAGAACUGAAUCCAUAAGGCGUA	gga-miR-146b-5p	2.09	1.67E-04	92.6	194.6
GUAGUGUUCCUACUUUAUGGA	gga-miR-142-3p	2.12	7.37E-04	57.3	121.9
UGAGAACUGAAUCCAUAAGGCGGU	gga-miR-146b-5p	2.08	1.49E-04	44.3	92.8
UGUCAGUUUGUCAAUACCCCA	gga-miR-223	2.68	6.04E-03	32.9	89.2
UGAGAACUGAAUCCAUAAGGCGUUC	gga-miR-146b-5p	2.32	2.99E-05	31.3	72.9
UGAGAACUGAAUCCAUAAGGCGUUUU	gga-miR-146b-5p	2.53	4.94E-05	26.9	68.1
UGUCAGUUUGUCAAUACCCCAU	gga-miR-223	2.47	8.45E-03	26.0	64.7

Note: The templated nucleotides in isomiR sequences are marked in dark shadow and the non-templated are in light shadow.

The test values including false discovery rate-adjusted *P* values (*P*<sub>adj</sub>) and foldchange (FC) are calculated by DESeq2 software, and the FC values are not numerically equal to MT:MC.

(FC = 0.77, *P*<sub>adj</sub> = 0.034) after infection, which was validated by qPCR result (0.72 times, *r* = 0.917), we deduced that miR-146b-5p might target and bind to *USP3* mRNA, repress its translation and reduce the deubiquitinase activity, which allowed the action of polyubiquitin chains of MDA5 and the activated RLR signaling, thereby leading to the continuous production of type I interferon. The upregulation of *MDA5* (FC = 3.63, *P*<sub>adj</sub> = 2.27E-06), which was validated by qPCR result (3.06 times, *r* = 0.963), might aim to complement its consumption and guarantee the signaling. It should be noted that *LGP2* also showed an increase after infection (FC = 2.44, *P*<sub>adj</sub> = 0.005), further hinting the activation of RLR signaling pathway because of the regulatory role of *LGP2* on this pathway (Rehwinkel and Gack, 2020). Additionally, there were three interferon genes in the RNA-seq data including type I interferon *IFNAL3* (FC = 0.89, *P*<sub>adj</sub> = 0.947) and *IFNKL1* (FC = 42.22, *P*<sub>adj</sub> = 0.009), and type II interferon *IFNG* (FC = 18.06, *P*<sub>adj</sub> = 3.33E-06). The deduced direction of miR-146b-5p action is consistent with that of type I interferon alteration, suggesting this regulation might be to sustain the immune state at 3 dpi.

In summary, we identified the factual upregulation of chicken miR-146b-5p at isomiR level in response to *S. Enteritidis* infection, and deduced that chicken miR-146b-5p might function to sustain RLR signaling and type I interferon induction by repressing *USP3* transcript.

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## DISCLOSURES

The authors declare that they have no competing interests.

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