MicroRNAs in the immune organs of chickens and ducks indicate divergence of immunity against H5N1 avian influenza

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A B S T R A C T

Chickens are susceptible to the highly pathogenic H5N1 strain of avian influenza virus (HPAIV), whereas ducks are not. Here, we used high-throughput sequencing to analyse the microRNA expression in the spleen, thymus and bursa of Fabricius of H5N1-HPAIV-infected and non-infected chickens and ducks. We annotated the genomic positions of duck microRNAs and we compared the microRNA repertoires of chickens and ducks. Our results showed that the microRNA expression patterns in the homologous immune organs of specific-pathogen-free (SPF) chickens and ducks diverge substantially. Moreover, there was larger divergence between the microRNA expression patterns in immune organs of HPAIV-infected chickens than HPAIV-infected ducks. Together, our results might help to elucidate the roles of microRNAs in the divergent immunity of chickens and ducks against H5N1 HPAIV.

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1. Introduction

The H5N1 subtype highly pathogenic avian influenza virus (HPAIV) originated from Asia has attracted global attention for more than a decade and has continuously posed a serious threat to the poultry industry as well as to public health [1]. Although H5N1 epidemic has been well controlled, concerns over this subtype of AIV remain due not only to its virulence in poultry, but also to its potential for inter-species transmission. Waterfowl, including domestic ducks, are considered to be the natural reservoirs of influenza A viruses [2], which are not affected by H5N1 viruses or show only milder signs of the disease. These infected birds can continue to shed and spread viruses through both their respiratory and intestinal tracts and play critical roles in the propagation and biological evolution of highly pathogenic H5N1 viruses [2]. Thus, ducks and other waterfowl represent the “Trojan Horses” of H5N1 avian influenza [3]. However, chickens are much more susceptible to H5N1 avian influenza.

Various studies have focused on the impact of a potential avian influenza epidemic in poultry due to the ever changing nature of avian influenza virus and on methods to increase antibody titres through vaccination to reduce morbidity and mortality [4,5]. However, only a few studies have explored the mechanism underlying the divergence of susceptibility across species at the levels of gene expression and post-transcriptional regulation [6]. Knowledge of multiple layers of regulation, including microRNAs (miRNAs), might provide insight into the immune regulatory network [7,8]. Variations in the miRNA/target repertoire have likely driven the phenotypic differences between related species [9].

In avian species, the thymus and bursa of Fabricius are central immune organs responsible for cell-mediated and humoral immunity, respectively. The spleen is an important source of effectors for both innate and cell-mediated adaptive immunity in response to influenza [10]. Therefore, in this study, we investigated the miRNAs expressed in these major immune organs, spleen, thymus and bursa of Fabricius, of chickens and ducks in the presence or absence of H5N1 HPAIV infection. The avian influenza virus

Abbreviations: H5N1 HPAIV, H5N1 subtype highly pathogenic avian influenza virus; SPF, specific pathogen free; CPM, count per million of reads
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mismatches. For both species, the sequencing reads in FASTA format were mapped to the respective genomes using Bowtie, allowing no parameters, with minor modifications of the scripts for ducks.

The animal experiments were conducted in bio-security level 3 laboratories. The workflow of grouping and sampling is illustrated in Fig. S1. Ten 1-month-old specific-pathogen-free (SPF) chickens (White Leghorns) and ducks (Anas platyrhynchos) were infected with the A/Duck/Anhui/1/2006 strain of avian influenza virus with a MOI of 10^6 EID<sub>50</sub>/bird for both SPF chickens and ducks. For each chicken and duck, 100 μL of virus was administered through nasal drops. On the 3rd and 7th days post-infection, which were the mean times of H5N1-HPAIV-infected chickens and ducks to die, respectively, these chickens and ducks, together with the SPF controls, were sacrificed. The spleen, thymus and bursa of Fabricius were collected (Figs. S2–4). Total RNA was immediately extracted using TRIzol (Invitrogen) following the manufacturer’s protocol. The RNA concentration and purity were determined by measuring the absorbance at 260 nm and the A260/280 ratio using a NanoDrop 2000 spectrophotometer (Thermo). The RNA samples from each tissue were pooled from three chickens or ducks. The sequencing libraries were prepared according to the Illumina Small RNA Sample Preparation protocol. The twelve libraries for chickens and ducks were each sequenced on one lane of a HiSeq 2000 sequencing system.

2.2. Detection of miRNA genes

Known miRNA precursors, mature sequences and miRNA families from chicken, mouse and human were retrieved from miRBase (Release 20). Duck genome assembly scaffolds were downloaded from NCBI. We then used miRDeep2 to annotate the known and novel miRNAs of both chickens and ducks [13]. As miRBase does not archive duck miRNAs, chicken and zebra finch miRNAs were used as the references. The sequences of the detected known miRNAs were compared with miRBase sequences to re-evaluate quality of our data. The miRDeep2 program was used with default parameters, with minor modifications of the scripts for ducks. For both species, the sequencing reads in FASTA format were mapped to the respective genomes using Bowtie, allowing no mismatches.

miRcute miRNA first strand cDNA synthesis kit and miRcute miRNA qPCR detection kit (Tiangen Biotech) were used for validation of the predicted novel miRNAs following the manufacturer’s protocols. U6 snRNA was used as the internal control. All reactions were run at least in duplicate. The qPCR was performed on an Agilent real-time PCR system.

2.3. Expression analysis

Bowtie was used to align reads from all libraries to the miRNA mature sequences using the following parameters: -n 0 –l 20 –a –best. The reads were required to start form exactly the 5' end and stop within three nucleotides downstream of the 3' end. To assess chicken and duck orthologous miRNAs, we classified the expressed miRNAs into different categories based on their evolutionary ages [14]. Ancestral miRNAs that are conserved in amniote species were selected.

Relative expression values [15] were used to evaluate miRNA expression across all tissues of chickens and ducks. The expression value for an individual miRNA was the normalised number of miRNAs divided by the sum of the miRNAs across all libraries for each species. Hierarchical clustering was implemented using the average linkage method. Heatmap of the expression levels was produced using R package “pheatmap”.

Differential expression analysis between all sample pairs was implemented using edgeR [16] in R. Filtering was implemented using the criterion of at least one CPM being found in at least 6 RNA libraries, and only miRNAs that were expressed in all tissues were retained. We then performed differential expression analysis using the exactTest in edgeR. The statistical significance was defined as follows: log 2 foldchange >1 and P < 0.05.

2.4. miRNA target prediction and functional analysis

The 3' UTR sequences of chicken miRNAs were downloaded from the UCSC Table browser and the Galaxy web-based platform (https://usegalaxy.org/). Duck 3' UTRs were retrieved on Galaxy using duck genome assembly scaffolds and genome coordinates downloaded from NCBI. Target prediction was performed with miRanda and PITA. The miRNA regulatory networks were constructed using cytoscape and the CyTargetLinker [17].

3. Results

3.1. miRNA repertoires in duck and chicken immune organs

Small RNAs from the spleen, thymus and bursa of Fabricius of chickens and ducks in the presence or absence of H5N1 HPAIV infection were subjected to high-throughput sequencing using HiSeq 2000. We obtained an average of 82 430 014 sequencing reads per library, with miRNAs accounting for 42.05–94.12% of these reads (median 88.71%, Fig. 1A and Table S6). The lowest miRNA coverage was observed in the spleen of infected chickens. The proportion of the duck reads mapped to miRNAs was estimated using chicken and zebra finch sequences. The number of unique reads corresponding to miRNAs ranged from 37 912 to 64 610 (median 48 156). These sequencing datasets were then analysed with two primary goals: to obtain miRNA repertoires and to analyse miRNA expression patterns in the immune organs of chickens and ducks with and without H5N1 HPAIV infection.

As duck miRNAs have not been annotated and archived in miRBase, we first sought to annotate the mature and precursor sequences of duck miRNAs. We detected the miRNAs by running the miRDeep2 pipeline [13]. The miRBase miRNAs of chickens and zebra finches were used as inputs. We were able to detect 269 mature duck miRNAs (Table S1). The method could also identify the duck miRNAs that were not previously annotated for chickens. For instance, the duck miRNA apla-mir-363-3p was predicted to be a homologue of zebra finch miRNA tgu-mir-363-3p belonging to the mir-106–363 cluster (Fig. S5). In addition, we predicted 222 novel miRNA loci for ducks at a miRDeep2 performance score cut-off >0, averaging 93 novel miRNAs per dataset (Table S2).

To make a comparison of our results with what was previously published on duck miRNAs, we ran the analysis pipeline on small RNA datasets of developing cranial neural crest [18]. A total of 249 miRNAs with chicken homologues were identified in these datasets, which were comparable to those in our datasets. As the novel miRNAs were predicted by algorithmic method, we validated the predicted chicken and duck novel miRNAs by real-time PCR.
(Fig. S6). Five chicken miRNAs that were not archived in the miRBase (Release 20) were confirmed by the experiment. Five novelly predicted duck miRNA could be detected by real-time PCR although three of them were expressed at low levels.

In chickens, 473 miRNAs were detected (Table S3), of which 413 miRNAs were annotated in miRBase (41.5%, out of all miRBase miRNAs). The percentage was low given the well-controlled data quality but was comparable to that in a previous study, averaging 466 (46.8%) miRNAs in the brain, cerebellum, heart and kidney [14]. In addition to the miRBase miRNAs, 267 novel miRNA loci with an miRDeep2 performance score cut-off >0 were also obtained (Table S4).

By combining the known and newly predicted miRNAs, we obtained miRNA catalogues for chickens and ducks. A total of 245 miRNAs were shared by the two species in these libraries, accounting for 98.5% and 99.9% of the sequencing reads that could be mapped to miRNAs from chickens and ducks. These results indicated that the miRNA repertoires of chickens and ducks are well conserved. Additionally, 211 miRNAs were specific to chickens, of which 175 were found in miRBase and 36 were novel or re-annotated miRNAs (Fig. 1B). Considering the existence of gaps in the sequenced genomes, the chicken and duck datasets were mapped to these miRNA sequences using Bowtie to confirm the species-specific expression. Of these 211 cases, 23 could be mapped with at least ten reads to at least one dataset in ducks. Finally, we confirmed 188 chicken-specific miRNAs. In addition, seven miRNAs were found to be specific to ducks although with low read numbers.

We showed above that the size of the duck miRNA repertoire was much smaller than that of chickens. Additionally, we grouped the duck miRNAs into 83 miRNA families versus 128 chicken miRNA families according to the miRBase definition. The evolutionary divergence between chickens and ducks is approximately 90–100 Myr [19], so the number of miRNA families gained by each species is estimated to be 30 given a gain rate for miRNA families of approximately 0.3 families/Myr in birds [14]. We propose here that the predicted novel duck miRNAs, except for the reliable and widely expressed species, may include some bona fide miRNAs that are lineage-specific. By aligning the predicted precursors to all hairpin sequences in miRBase, we obtained hits with an identity >70% and an e-value <10^{-5} belonging to 6 miRNA families. However, the other 213 precursors could not be classified into any of
the known miRNA families. We also identified 10 novel miRNA families in ducks from the predicted novel precursors, of which 3 families were specific to chickens. It might be expected that the number of species-specific miRNAs in ducks was comparable to that in chickens. Thus, the low number reported here might reflect the fact that the duck miRNAs were being predicted de novo and the gaps in the duck genome assembly.

### 3.2. The miRNA expression divergence of SPF chickens and ducks

Most of the miRNAs identified to date were previously assumed to be expressed in a tissue-specific manner and to be highly conserved across animal phyla [20]. However, later research suggested that the temporal and spatial patterns of miRNA expression are not strictly conserved between species [21]. And different tissues did not experience the same amount of changes in miRNA expression and the regulatory networks [8]. Thus, comparison of miRNA expression in the homologous immune organs of chickens and ducks might provide information about the observed divergent immunity to H5N1 highly pathogenic avian influenza.

To directly compare the miRNA expression in chickens and ducks, it was necessary to choose a subset of miRNAs and normalise their expression levels. Since recently evolved miRNAs usually have low expression levels and limited regulatory functions, we selected the ancestral miRNAs that originated before birds as reported previously [14]. A total of 116 mature miRNAs fell into this category. The expression level for a miRNA in each library was normalised as the read counts per million of reads matching miRNAs in that library (RPM). Ratios of normalised miRNA counts of ducks to that of chickens was defined as the expression divergence.

Although these miRNAs are conserved at the DNA level, there was large expression divergence in the three organs between chickens and ducks. Each of the organs displayed a number of differentially expressed miRNAs as shown in the 3D scattered plot and the heatmap (Fig. 2). And the diverged miRNA expression was distinct for each of the organs. Of the 116 conserved miRNAs, respectively, 17, 14 and 23 miRNAs were shown to be more abundantly expressed in the spleen, thymus and bursa of Fabricius of ducks than those of chickens (log 2 foldchange >1). Additionally, expression levels of 28, 21 and 20 miRNAs were lower in duck immune organs than in those of chickens (log 2 foldchange <−1) (Table S5). For example, miR-122-5p, which was found to be highly expressed in lungs of HPAI-v-infected broilers versus the non-infected ones and suggested to be involved in host response to HPAI infection [22], was much more abundantly expressed in the spleen and bursa of Fabricius of ducks than in those of chickens (log 2 foldchange = 5.01 and 2.14) but lower in the chicken thymus than in that of ducks (log 2 foldchange = −4.44). Among other miRNAs expressed in species- and tissue-specific manners, some with high abundance were marked in the scattered plot (Fig. 2A). Although we could not associate many of the differentially expressed miRNAs with their functions and the divergent immunity, it would be beneficial to our understanding of the divergence of regulatory mechanisms in the homologous immune organs of chickens and ducks.

### 3.3. H5N1 HPAI induced distinct miRNA expression changes in the immune organs of chickens and ducks

To present the expression profiles across all the chicken and duck immune organs, we calculated the relative expression level of a miRNA as the percentage of its normalised count across the six libraries of chickens or ducks. Then hierarchical clustering was performed on the relative miRNA expression levels in all the libraries by Pearson correlation method (Fig. 3A). The clustering revealed that each of the immune organs of chickens and ducks had marked signatures in their miRNA expression levels, which could separate them from each other. Interestingly, miRNA expression in the spleen and bursa of Fabricius of infected chickens more resembled that of SPF ducks, while the expression patterns of SPF chickens more resemble those of infected ducks. However, miRNA expression in the thymus of infected chickens was distinctly different from that in thymuses of SPF chickens and ducks (Pearson correlation, \( r = 0.0324 \) with SPF chickens, and \( r = 0.1100 \) with infected ducks). And miRNA expression in the thymus of SPF chickens was highly correlated with those of ducks (Pearson correlation, \( r = 0.5588 \) with SPF ducks, and \( r = 0.4949 \) with infected ducks). The data suggested that the miRNA regulatory networks in the thymus of infected chickens were severely altered.

Differential miRNA expression between each pair of tissues of infected and SPF chickens and ducks was then determined by the edgeR method [16]. Overall, there were greater miRNA expression changes in chicken immune organs than in those of ducks (Fig. 3B).
Among the 116 conserved miRNAs, respectively 27, 39 and 26 miRNAs were shown to be up-regulated in the spleen, thymus and bursa of the Fabricius of infected chickens versus the SPF chickens (log 2 foldchanges >1, \(P<0.05\)), while 10, 19 and 16 miRNAs were down-regulated (log 2 foldchanges < -1, \(P<0.05\)) (Tables S7–9). In the duck immune organs, however, only 8, 6 and 9 miRNAs were respectively down-regulated in the three immune organs (log 2 foldchange < -1) (Tables S10–12). The numbers of up-regulated miRNAs were even smaller. Thus, we revealed that miRNA expression changes in the immune organs of chickens and ducks infected by H5N1 HPAIV were greatly diverged, which paralleled to their divergent susceptibility to H5N1 HPAIV.

4. Discussion

In this study, we first annotated duck miRNAs using high-throughput sequencing and bioinformatics methods. We obtained miRNA repertoires and expression levels in the three major immune organs of chickens and ducks, i.e., the spleen, thymus and bursa of the Fabricius, in the presence or absence of H5N1 HPAIV infection. Second, we revealed that there was large divergence in miRNA expression between homologous immune organs of SPF chickens and ducks. Each of the immune organs displayed a unique feature of species-specific expression, indicating the difference in miRNA regulatory networks of the two species. Third, we elucidated the global miRNA expression changes in the spleen, thymus and bursa of the Fabricius of chickens and ducks after H5N1 HPAIV infection via expression analysis.

Phenotypic differences between species are shaped by patterns of gene expression, which are themselves regulated at many levels [23]. The evolution of organisms is accompanied by the increasing complexity of multi-layered gene regulation mechanisms [24]. Specifically, miRNAs have been identified as a causal factor [25]. In primates, changes in miRNA expression were shown to contribute to evolution of cognitive functions [21]. In this study, we found that there were divergences in both the miRNA repertoires and miRNA expression patterns in the immune organs of SPF chickens and ducks. In spite of the conservative nature of the selected miRNAs, their expression in the homologous immune organs showed large divergence. Further evaluation of the roles of these miRNAs in the development of immune organs of chickens and ducks will provide useful information about the divergent immunity against H5N1 HPAIV.

By hierarchical clustering and differential expression analysis, we identified the global miRNA expression patterns in the immune organs of HPAIV-infected SPF chickens and ducks and obtained the differentially expressed miRNAs with HPAIV infection. The results were in agreement with the clinical manifestations of the two avian species with H5N1 HPAIV infection. Experimentally, HPAI viruses typically produce a severe and systemic disease with high...
mortality in chickens and other gallinaceous birds [26]. In addition to the severe and consistent gross lesions including splenomegaly and pulmonary oedema and congestion, histologically, moderate to severe lymphoid depletion with apoptosis to necrosis in remaining lymphocytes were observed in the spleen, thymus and bursa of Fabricius of H5N1-HPAIV-infected chickens [27]. Functions of these organs might be severely disturbed. For ducks, we observed no obvious change in the gross lesions in duck immune organs. Thus, the divergent miRNA expression changes of infected chickens and ducks reflected the different status of infection. Of the selected conservative miRNAs, more miRNAs were up-regulated in immune organs of infected chickens than the down-regulated ones, which was consistent with a previous study in lungs of avian influenza virus infected broiler chickens [22]. However, in immune organs of infected ducks, the number of up-regulated miRNA was smaller than that of down-regulated ones. The differences in miRNA expression between chickens and ducks would have great impact on the expression of target genes in pathways related to immune response. To understand the miRNA expression divergence, we predicted the miRNA regulatory networks of chickens and ducks. For example, B cell receptor (BCR) signalling pathway controls proliferation and differentiation of B cells and antibody production.
In the spleen of infected chickens, the up-regulated miRNAs, gga-miR-2188-5p, gga-miR-34c-5p, gga-miR-200b-5p, gga-miR-122-5p and gga-miR-146b-5p, were predicted to target the genes in the BCR signalling pathway (Fig. 4A). However, in the spleen of infected ducks, only one down-regulated miRNA, apla-miR-122-5p, was predicted to target RASGRP3, which, on the contrary, would contribute to BCR signalling (Fig. 4B). Therefore, the divergent miRNA expression changes in the immune organs of chickens and ducks infected by H5N1 HPAIV would have great influence on the different susceptibility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.12.019.

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