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Research Article

First Cloning, Tissue-specific Expression and Molecular Characterization of RIG-I Gene in Whooper Swan

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Abstract

To investigate the molecular characteristics of the RIG-I gene of whooper swan, RIG-I gene was cloned, sequenced and characterized, and the relative expression levels of the RIG-I gene in five tissues of whooper swans were determined by Real-time Quantitative PCR (qPCR). The RIG-I gene of whooper swan has a 2964 bp CDS that encode a peptide of 987 amino acids, RIG-I is widely expressed in various tissues of the whooper swan, but the expression levels show certain differences. The homology analysis indicated that the RIG-I gene of whooper swan has a more than 90% homology with other *Anseriformes* birds including mallard, swan goose, black swan and mute swan. Phylogenetic analysis showed that RIG-I gene of whooper swan and other birds have grouped in a separate branch. Gene alignment analysis revealed that RIG-I gene of swan has an extra low complexity region compared with that of non-swan birds. The amino acid alignment analysis showed that RIG-I of swan has a specific S62G phosphorylation site mutation compared with that of other birds and ubiquitination/phosphorylation mutation sites (RNF122, CK2, and REUL I) which differ from that of human. This is the first cloning and characterization of RIG-I gene from whooper swan. It may enhance our understanding of the molecular response mechanism of RIG-I against the influenza virus.

Keywords: Cloning, Molecular characterization, RIG-I, Tissue-specific expression, Whooper swan

Introduction

Whooper swan *(Cygnus cygnus),* belonging to the *Anseriformes Anatidae Cygnus*, are widely distributed worldwide waterfowl. They breed in the Palearctic and range from Iceland to the Far East, across Eurasia, and are widely distributed in freshwater regions [1]. Whooper swan, due to their long-distance migratory habits, are often in contact with other birds, which increases their risk of contracting Avian influenza viruses (AIV) [2]. During the H5NX avian influenza pandemic, swans were reported to be infected and die. It makes swan considered as indicators of the H5 subtype of AIV [3,4]. In recent years, H5 avian influenza has evolved into new varieties, which threaten poultry farming and human health [5]. Whooper swan are more sensitive to influenza viruses than other waterfowl [6]. The mechanism behind this phenomenon has attracted great attention from scholars in the field.

Retinoic Acid Inducible Gene-I (RIG-I) is a key intracellular pattern recognition receptor that plays an important role in recognizing and responding to RNA viruses such as avian influenza [7,8]. Barber's study [9] showed that ducks have a natural resistance to avian influenza while chickens are highly susceptible to avian influenza infection. It is strongly associated with ducks having an intact RIG-I gene and chickens lacking it. RIG-I, as a cytoplasmic RNA sensor, is able to respond to infection with influenza viruses, trigger IFN-β production and activate downstream IFN-stimulated antiviral gene expression [10]. Ducks possess a complete RIG-I gene, enabling them to induce IFN production and downstream antigen expression via RIG-I, thus exhibiting natural resistance to AIV [9,10]. On the other hand, Chickens lack the RIG-I gene, although it can express IFN-α by other pathways the absence of RIG-I-mediated IFN-β severely compromises antiviral defense, making them highly susceptible to

AIV [9,11]. Previous experimental studies in Muscovy ducks *(Cairina moschata)* [12], geese [13] and pigeons *(Columba livia)* [14] also confirmed the presence of the RIG-I gene and successfully validated its mediated IFN-β expression and its protective role in antiviral immunity, further supporting the critical immunoprotective functions in different avian populations.

There is currently no research confirming whether the susceptibility of whooper swans to AIV is related to the RIG-I gene, and no study has confirmed whether whooper swans possess the RIG-I gene. Genbank predicted the RIG-I sequences of black swan and mute swan by genome sequencing. However these predictions have not been experimentally validated. In this study, it designs primers based on reference prediction sequences, attempts to amplify the complete gene sequence of the Giant Swan RIG-I, analyzes its molecular characteristics and predicts its spatial structure and function. It will explore the possible relationship between the integrity and function of RIG-I gene and the high susceptibility to avian influenza from the perspective of immunology. It can provide reference and guidance for the effective prevention and control of avian influenza.

Material and Methods

Ethical Statement

This study was approved by the Northeast Forestry University Animal Experiments Local Ethics Committee (Approval no: 2023004).

Experimental Animal and Sample Collection

Samples were collected from the fresh carcass of a whooper swan that succumbed to canine bite injuries despite treatment at the Sanmenxia Wildlife Rescue and Monitoring Center in Henan Province, China. The carcass tested negative for infectious diseases. The lung, liver, spleen, kidney, larynx, and rectum tissue was milled with liquid nitrogen to form homogenates and stored in -80ºC for later use.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the stored tissue according to the operation instructions of the Eastep® Super Total RNA Extraction Kit (Promega, Madison, America). The purity and concentration of the extracted RNA were determined to use a photometer. CDNA was obtained by reverse transcription of RNA using the PrimeScript™ RT reagent Kit (Takara, Otsu Shiga, Japan).

Sequence Identification and Molecular Cloning

According to the predicted *Cygnus olor* (XM_040540509.1) of RIG-I sequence in Genbank, the primer design software Oligo7 was used to design the amplified full length sequences *(Table 1)*. PCR amplification was performed using the high-fidelity enzyme PrimeSTAR® Max DNA Polymerase (Takara, Otsu Shiga, Japan) with cDNA of Cygnus swan lungs synthesized by the above steps as a template and RIG-I-F/R as a specific primer. PCR products were recovered and purified using Takara MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Takara Bio Inc, Kusatsu, Japan), the purified product was subcloned into the PMDTM19-T vector (Takara, Otsu Shiga, Japan), the recombinant was transformed into *E. coli* Dh5α, and the plasmid was extracted according to the instructions of Plasmid Purification Kit (Midi) (Abnova, Taiwan, China). Finally, the recombinant plasmid was identified by PCR. Three recombinant plasmids, which were identified as positive by PCR, were randomly selected and sent to biotechnology companies for sequencing. The sequencing results were confirmed by BLAST on NCBI *(https://www.ncbi.nlm.nih.gov)* with RIG-I genes from different species [15].

Table 1. *The primer for PCR amplication in this study*

Primer	Primer Sequence (5'-3')	Product Length
RIG-I	F: ATGAGGTTCACGAAGCTGCAAGC R: CTAAAATGGTGGGTACAAGTTGG	2964bp

Bioinformatics Analysis of RIG-I Gene

The homology of the RIG-I gene of whooper swan with that of humans and 16 other animals was compared by Clustau W Method using Meglign software, with sequence information from the NCBI database *(https://www.ncbi.nlm.nih.gov/)*. Using the N-J method of MEGA7.0 software, the phylogenetic relationships and taxonomic positions of RIG-I genes in birds, fish and mammals were observed and analyzed, and used ITOL's online tool *(https://itol.embl.de)* to beautify the modified gene tree [16]. The RIG-I amino acids of whooper swan, humans, pigeons, mallards and swan geese were compared by GeneDoc software. The proteins transcribed by the RIG-I gene of swan were predicted by a genome-wide database search in SMART *(http://smart.embl-heidelberg.de)*, and the hydrophobic profile of RIG-I was predicted by the ProtScale program of ExPASy; used the online tool SignalP *(https://services.healthtech.dtu.dk/service.php?SignalP)* to predict protein signal peptides.

qPCR

The expressions of RIG-I gene in lung, liver, spleen, kidney, larynx, and rectum of whooper swan were detected using relative quantitative PCR. Each tissue sample was tested in triplicate and the average value was taken. Q-PCR was conducted using the TaKaRa SYBR® PrimeScri™ RT-PCR kit (TaKaRa, Beijing, China) according to the manufacturer's instructions [15]. The details of the primer sequences for qPCR are listed in *Table 2*. The cDNA template was from the above experiment.

Table 2. The primer for PCR amplication in this study			
Primer	**Primer Sequence (5'-3')**	**Product Length**	**Reference Sequence**
qRIG-I	F: TGACATCATCGTACTGACACC R: CAGCTGACTTGCAGAGGAGTT	184bp	XM_040540509.1
qGAPDH	F:TTGGCATCGTGGAGGGTCTTATG R:CCCGTTGAGCTCAGGGATGACTT	176bp	XM_040573143.1

Statistical Analysis

With GAPDH gene as the internal reference, the expression level of RIG-I gene in liver was set to 1, the relative expression levels of RIG-1 gene in different tissues were analyzed by $2^{-\Delta\Delta Ct}$ method. Statistical analysis and data plotting were performed using GraphPad Prism 10.1.2 (GraphPad Software Inc.) [17].

Results

Cloning of the RIG-I Gene

Using lung cDNA as a template and RIG-I-F/R as specific primers, the full-length RIG-I was amplified. The PCR amplification products of RIG-I gene fragments of the mute swan were about 2900bp in size, as shown by 1% agarose gel electrophoresis *(Fig 1-A)*. It was consistent with the expected results. The sequencing results showed that the full-length sequence of the cloned RIG-1 gene is 2964 bp, which has high homology with several avian species in NCBI.

Bioinformatics Analysis

The mRNA of RIG-1 in different tissues of the whooper swan was detected by qPCR, with GAPDH as the internal reference. Expression in different tissues was calculated. The qPCR results showed that the RIG-1 gene was expressed in all the tested tissues, with a high level in lung, spleen and kidney, and moderate level in larynx and rectum, but low level in liver *(Fig 1-B)*.

Using Megalign software, the RIG-I gene sequences of whooper swan were compared with those of other birds, mammals and fish *(Fig 2-A)*. The RIG-I gene sequences of whooper swan were found to be 98.9% homologous to those of swan goose *(Anser cygnoides)*, black swan *(Cygnus atratus)* and mute swan *(Cygnus olor)* by comparison. Therefore, the amplified whooper swan RIG-I gene of length 2964bp is matched with swan goose, black swan and mute swan.

The amino acids of RIG-I of whooper swan were compared with those of humans, pigeons, mallard ducks and swan geese by GeneDoc software. The gene sequences

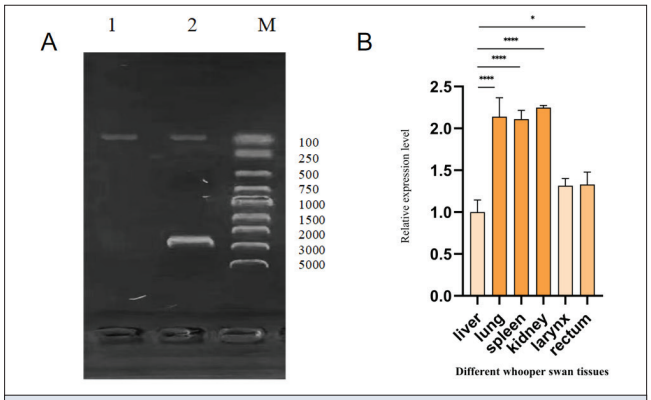

Fig 1. PCR amplification of RIG-I gene and its quantitative distribution in various tissues. **A:** PCR amplification results of RIG-I gene of whooper swan. The first lane is the negative control, the second lane is the amplified fragment, and the M lane is the DL5000 DNA marker, **B:** Relative gene expression patterns of RIG-I in various tissues of swan. The GAPDH was used as internal control. Bars represents the mean ± SD (n=3). * stands for P<0.05, **** stands for P<0.0001. It is considered that P<0.05 is statistically significant

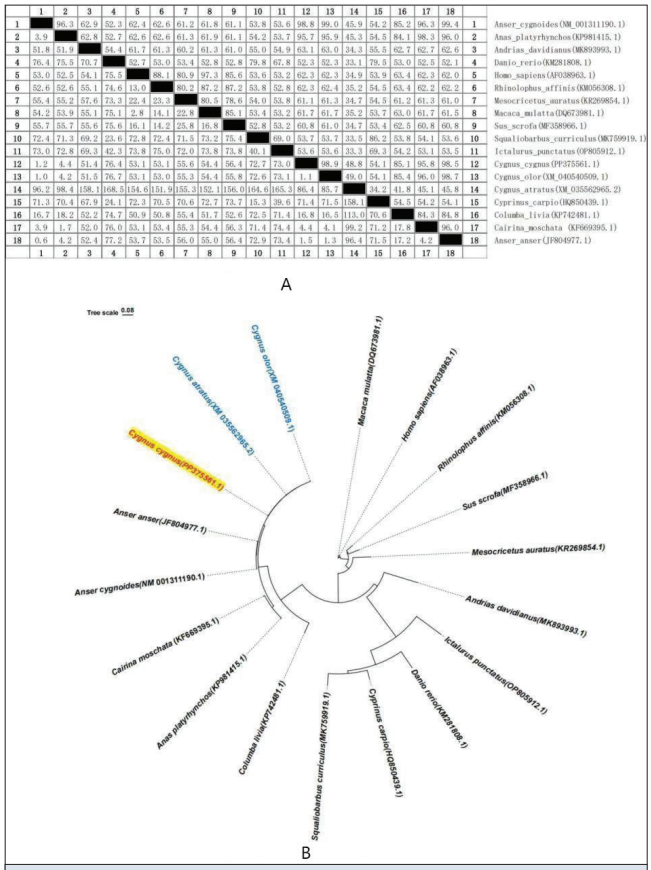

Fig 2. The homology and phylogenetic analysis of RIG-I from whooper swan and other species. **A:** The homology analysis of RIG-I gene from some birds, human, mammals and fishes, **B:** Phylogenetic analysis of RIG-I from some birds, human, mammals and fishes. The blue font represents the predicted RIG-I gene of *Cygnus atratus* and *Cygnus olor*, while the red font and yellow background represent the RIG-I gene of *Cygnus cygnus*

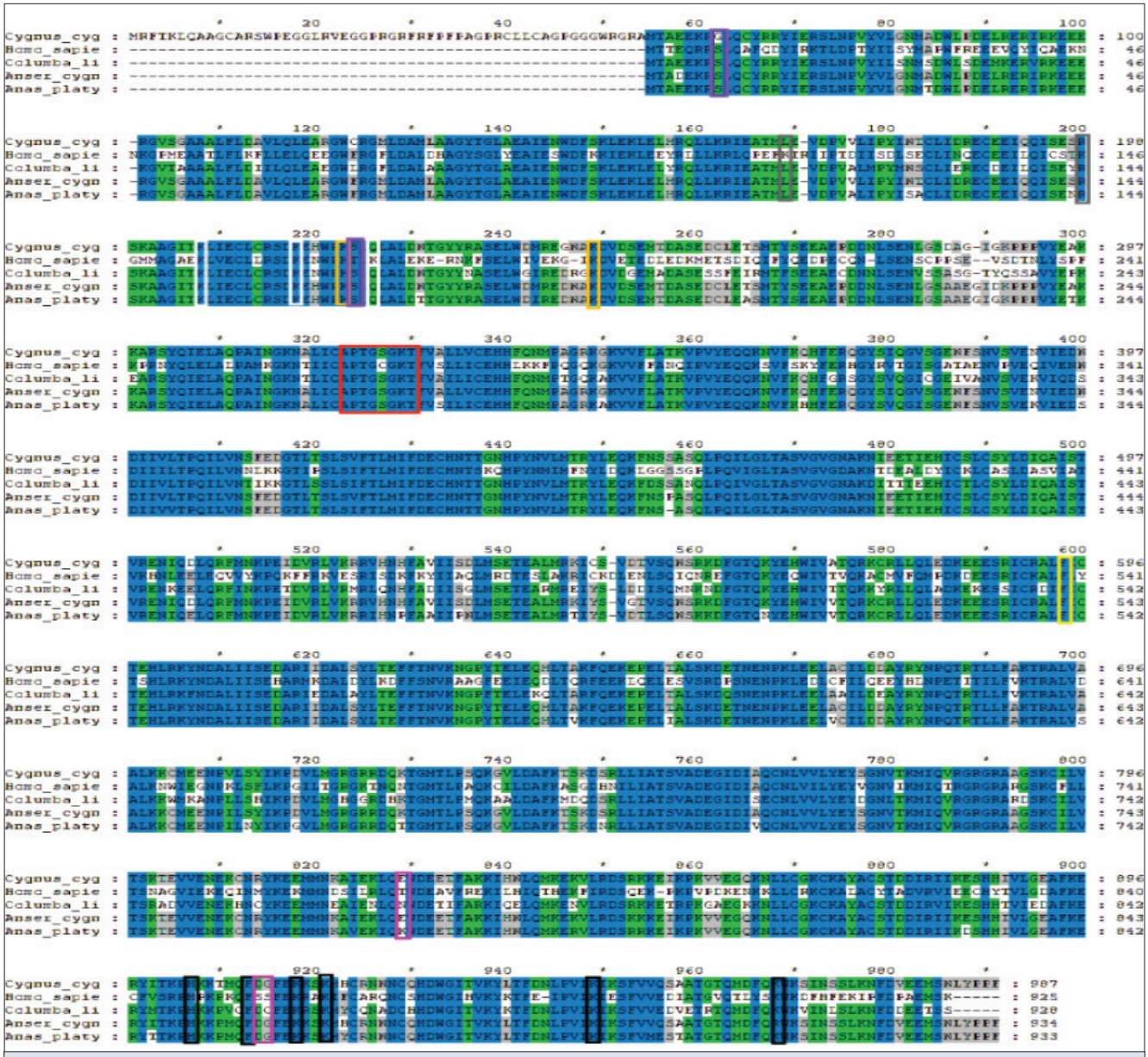

Fig 3. Amino acid alignment of Cygnus Cygnus, Homo sapiens, Columba Livia, Anser cygnoides and Anas platyrhynchos RIG-I. The purple frame represents the phosphorylation site of PKC-α/β. The pink frame denotes the phosphorylation site of CK2. The orange frame indicates the ubiquitination site of TRIM25. The white frame represents the REUL ubiquitination site. The gray frame denotes the ubiquitination site of RNF122. The yellow frame indicates the key residues of the interaction between CARDs and HEL2i. The red frame represents the ATP-binding motif, and the black frame denotes the key residues of RNA ligand binding

of black swan and mute swan were predicted based on the macro-genome sequences, but they were not confirmed by experiments. The starting part of the RIG-I amino acid sequence of whooper swan in *Fig 3* is significantly longer than that of other species. However, the biological significance of this amino acid variant requires further investigation due to limited data.

The amino acid sequence of RIG-I was predicted by SMART, an online tool. The results showed that RIG-I had a classical structure, that is two CARD regions, one DExD/h helicase region and one C-terminal repression domain (RD). However, compared with the traditional RIG-I domain, there is a low complexity region (LCR) at the N-terminal of the swan RIG-I, starting at position 24 and ending at position 39, it may not have a significant effect on the function of the RIG-I gene. There are two CARD domains in the great swan RIG-I protein domain. The first starting at position 55 and ending at position 146, and the second starting at position 152 and ending at position 243. The DEXDc frame RNA enzymatic helicoid structure starts at position 295 and ends at position 505; the RD domain starts at position 862 and ends at position 980, so the swan RIG-I has a more typical RIG-I structure. RIG-I protein was bioinformatics by an online program

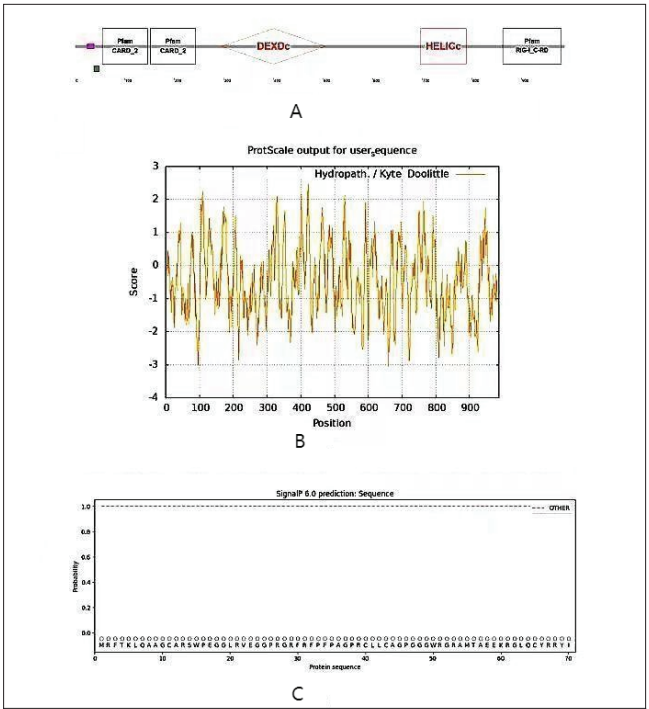

Fig 4. Structure and function prediction diagram of RGI-I proteins of whooper swan. **A:** Whooper swan RIG-I protein domain prediction, **B:** Whooper swan RIG-I protein hydrophobicity prediction, **C:** Whooper sawn RIG-I protein signal peptides prediction

(Fig 4-A). The results indicated that RIG-I protein was a hydrophilic stable acidic protein without signal peptide, so it was not a secretory protein *(Fig 4-B,C)*.

Discussion

The qPCR results of this study indicate that the RIG-I gene in whooper swan exhibits high expression level in the kidney, lung, spleen, rectum and larynx. Unlike duck, muscovy duck and goose where it is highly expressed, RIG-I gene expression in the liver of whooper swans is at a relatively basal level [12,16,18]. The elevated expression levels of RIG-I in the liver of waterfowl, as observed across multiple species, provide compelling evidence for the significant role of RIG-I in the innate immune response to AIV, supporting the possibility of respiratory transmission. And the high expression levels in the larynx and rectum of whooper swan suggest that this species can be infected with AIV via the digestive system [19]. May be the higher RIG-I gene expression level in lung and spleen of the whooper swan may allow it to be infected and die later thus providing a longer period for spreading AIV [20]. However, limited by the small sample size, this study lacked transfection, Western blot analysis and viral infection experiments compared to the studies mentioned. Consequently, the findings of this study can only be interpreted with caution,

In non-avian animals, the RIG-I gene exhibits variations: some animals with deleted RIG-I genes rely on the melanoma differentiation-associated protein 5 (MDA5) gene, a member of the RLR family, to fulfill its role. For instance, the Chinese tree shrew *(Tupaia belangeri chinensis),* which lacks the RIG-I gene, utilizes its MDA5 gene to recognize not only the small nuclear RNA virus originally specifically recognized by RIG-I, but also the Sendai virus of the *Paramyxoviridae* [21,22]. In addition, some animals lack the RIG-I gene-induced IFN pathway. Instead they play a role through other proteins. Also, IFN and MAVS transcripts that help induce IFN have not been detected in lampreys, according to Ma et al.[15] and Lu et al.[23]. Scientists have conducted RIG-I gene amplification experiments on the black flying fox to investigate its role in asymptomatic infections with multiple RNA viruses. These studies aim to understand how RIG-I contributes to the immune response and why black flying fox can carry RNA viruses without showing symptoms, and finally came to the conclusion that the RIG-I gene is intact and has obvious functions in bats [24]. Based on this research background with regard to our experiment, can this explain that the RIG-I gene is not the reason why the whooper swan is susceptible to avian influenza.

The RIG-I gene sequences of the black swan and the mute swan were predicted based on metagenomics, so the sequencing results are for reference only. Compared with *Anser cygnoides*, *Anser anser*, *Anas platyrhynchos*, and *Cairina moschata*, *Columba livia*, RIG-I gene has high homology *(Fig 2-A)*. Compared with mammals, the homology of the RIG-I gene sequence ranged from 61.3% to 62.3% in whooper swan and ranged from 52.5% to 54.4% in fish. It indicated that RIG-I gene had obvious interspecific characteristics. This result is similar to previous reports on the RIG-I gene in mallard ducks [25]. In the evolutionary tree *(Fig 2-B),* the RIG-I gene of *Columba Livia* (GenBank: KP742481.1), which belongs to the same class of birds, was less homologous to the RIG-I gene of whooper swan. Compared with mammals and fishes, there are obvious branches of birds. It can be seen that there are great differences in homology between different species.

The mechanism of RIG-I resistance to avian influenza is that directly interacts with the nucleocapsid of influenza viruses to activate RIG-I. When the PB2 proteins of avian influenza have lower affinity for NP proteins, the exposure of 5' PPP dsRNA to RIG-I is enhanced. Because the residues H904, F910, K915, K918, K946 and K965, which are necessary for the binding of the c-terminal domain of 5' PPP dsRNA, are completely conserved in swan [26] *(Fig 3 black frame)*. RIG-I has the ability to sense avian influenza RNA. RIG-I adopts a 'closed' conformation at resting state, which shifts to an 'open' structure upon binding viral RNA, thereby exposing its CARD domai [27]. The residue F595 required for the tight connection between its

CARD and Hel2i is conserved in order to remain silent *(Fig 3 tellow frame)*. Walker A's ATP-binding motif *(Fig 3 red frame)* is also conserved in the RIG-I gene of swan as a ligand-dependent ATPase. Upon viral infection, RIG-I remains active, and RIG-Is' ubiquitination is induced by TRIM25 can effectively help RIG-I-MAVS interactions [28], ultimately initiating antiviral IFN responses. In birds such as mallard ducks and swan geese, the S8 residues of RIG-I proteins are phosphorylated by protein kinase c-α/β (PKC-α/β), which helps to maintain the inactive state of RIG-I. However, this mechanism in swan is changed, with the S62 residue being replaced by G62. It may have affected the phosphorylation of PKC-α at this site and altered the mode of active regulation of RIG-I. It makes RIG-I of swan less active for IFN induction, but the specific mechanism of effect requires further investigation. Whooper swan S222 (wS222) residues are conserved, suggesting that the pathway might be involved in different residues in different birds *(Fig 3 purple frame)*. Residues K221 and K247, ubiquitylated by TRIM25, are also present in duck RIG-I *(Fig 3 orange frame)*.

PKC-α/β plays an important regulatory role in RIG-I-mediated type I IFN responses. Conventional PKC-α-β-induced RIG-I phosphorylation and TRIM25-mediated RIG-I ubiquitination functionally antagonize each other to tightly regulate RIG-I CARD-mediated antiviral signaling. Although other pathways can produce IFN-α and IFN-β, type I interferon expression after avian influenza infection is largely dependent on RIG-I [29]. Studies have shown that TRIM25 can bind to the CARD domain of RIG-I [30,31], and REUL has a homologous domain pattern and regulates RIG-I in a similar manner. Unlike TRIM25, REUL mediated K154, K164, and K172 residues, and when CK226 residues were mutated, REUL-mediated ubiquitination was attenuated and the ability of RIG-I to induce antiviral signaling was attenuated [32] *(Fig 3 white frame)*. The T770, S854/855 residues in the RIG-I c-terminal domain are critical for phosphorylation regulation of human casein kinase II, but these residues are absent from RIG-I in swan. Thus, there is no process by which cells at rest are phosphorylated by casein kinase II (CK2), and silencing of CK2 enhances RIG-I-mediated antiviral effects compared with normal condition [33] *(Fig 3 pink frame)*. RNF122 interacts with the CARD domain of RIG-I, promoting the K-48 ubiquitin chains at K115 and K146 of the CARD domain and leading to degradation. In birds, both key sites are mutated so that there is no degradation of RIG-I [34] *(Fig 3 gray frame)*.

A significant limitation of this study is the small sample size, stemming from substantial challenges in sourcing suitable specimens. Our original plan required at least three healthy, fresh whooper swan samples to achieve the dual objectives of amplifying the RIG-I gene and investigating its expression profile across various tissues and organs. Procuring such samples, however, proved exceptionally difficult. Since the whooper swan is listed as a national Class II protected animal in China, it is illegal to obtain samples through hunting. During field collection, a significant number of individuals encountered succumbed to infectious diseases, potentially compromising the detection of RIG-I gene tissue expression. Therefore, it cannot be used in this experiment. This sample comes from the vicinity of the Sanmenxia Wildlife Rescue and Monitoring Center. Local residents discovered a stray dog attacking a large swan and immediately reported it to the center. Upon receiving the report, the rescue team swiftly rushed to the scene, but upon arrival, they found that the large swan had unfortunately died. Further analysis at the facility verified the non-detection of significant infectious diseases, including Avian influenza and Newcastle disease, with all other test results coming back negative. The collaborative agreement we signed with multiple national wildlife reserves may potentially allow us to access healthier and fresher specimens in the future. However, achieving this goal in the short term is not feasible. Once we obtain such quality specimens, we will replicate this experimental study to validate the findings.

This study first confirmed that the whooper swan carries a functionally intact RIG-I gene (the sequence has been submitted NCBI, GenBank: PP375561.1). It filled the blank of the lack of experimentally validated RIG-I gene sequences swan species and systematically analyzed its molecular characteristics and functions. Sequence analysis showed that the whooper swan RIG-I had an additional LCR compared with other reported avian RIG-I genes. Notably, based on the genomic prediction, the RIG-I sequences of the black swan *(Cygnus atatus)* and the mute swan *(Cygnus olor)* also contained a similar LCR [12-14,16], however, this finding was excluded from the discussion owing to the absence of experimental validation. Structural variants, known to influence genetic diversity and susceptibility to diseases in various organisms, might similarly impact the whooper swan's vulnerability to AIV. A specific amino acid mutation site S62G was discovered that the mutation might adversely affect the RIG-I-mediated immune response to AIV. Protein structure prediction revealed that the RIG-I of the whooper swan possesses the typical features of RIG-I structural domain composition, indicating that it has the basic molecular architecture as a pattern recognition receptor. However, limited by the status of the whooper swan as a national Class II protected animal in China, this study only obtained a single effective sample, resulting in insufficient sample size. We will actively supplement samples in the follow-up research to further verify the current findings.

Declarations

Availability of Data and Materials: The original data of the paper are available upon request from the corresponding author.

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