Short Communication

Avian Sarcoma/Leukosis Virus (RCAS)-mediated Over-expression of IFITM3 Protects Chicks from Highly Pathogenic Avian Influenza Virus Subtype H5N1

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Abstract

Broad-spectrum antiviral activities of interferon-induced transmembrane proteins (IFITMs) are primarily attributed to *in vitro* inhibition of viral entry. Here, we used an avian sarcoma-leukosis virus (RCAS)-based gene transfer system and successfully generated chicks that constitutively express chicken IFITM3 (chIFITM3). The chIFITM3-overexpressing chicks showed significant protection and disease tolerance against highly pathogenic avian influenza virus (HPAIV) H5N1 (Clade 2.2.1.2). The chicks, overexpressing chIFITM3, also showed delayed onset of clinical symptoms, reduced viral shedding, and alleviated histopathologic alterations compared to control and challenged chicks. These findings highlight that overexpression of chIFITM3 provide a substantial defense against zoonotic H5N1 *in vivo*.

1. Introduction

Influenza A virus (IAV) is one of the most common viral infections in human and mammalian populations [1]. During IAV entry into the cellular compartments, the viral and cellular cues are delivered in a stepwise manner [2]. Upon entry, host innate immune responses exert the first line of defense against IAV infections [3] which are mainly attributed to the induction of interferon (IFN) [4]. There are three types of IFNs and when triggered, they induce numerous molecular changes including cell growth and inflammation [5]. The interaction of IFNs with their cognate receptors on the cell surface results in the activation of interferon-stimulated genes (ISGs) genes through IFN-stimulated response elements (ISRE) and gamma-activated sequence (GAS) promoter elements [6].

Interferon-induced transmembrane protein 3 (IFITM3) is a widely expressed ISG that has a known function of inhibiting the replication of pathogenic viruses including IAVs (4). Human IFITM3 was first identified using two genome-wide screens of RNAi and yeast-two-hybrid as a host restriction factor against human IAV [7]. Mechanistically, IFITM3 is believed to block the fusion of IAV virus with target cells at the stage of hemifusion and/or fusion pore formation by reducing membrane fluidity or by increasing the spontaneous positive curvature of the outer membrane [8]. While human IFITM3 has extensively been characterized both *in vitro* and *in vivo*, chicken IFITM3 (chIFITM3) remained poorly characterized especially when chicken and human IFITM3 only share a 42% amino acid identity (23).

On the other hand, IAVs represent a high level of genetic and functional diversity. The clade 2.2.1.2 of the highly pathogenic avian influenza virus (HPAIV) subtype H5N1 is globally distributed among wild and domestic birds with a higher evolution rate (6.9×10^{-3} substitution/site/year) and noticeable pathogenicity (24). Due to the

continued evolution of the virus, vaccines offer time-limited efficacies. Therefore, there is an unmet need to explore alternative options in establishing host factor-driven protection against viral infections.

In the current study, we aim to investigate the roles of chIFITM3 against HPAIV H5N1 clade 2.2.1.2 in developing chicks. The findings demonstrate the potential of chIFITM3 in not only reducing mortality but also alleviating clinical outcomes of the infection in chicks, highlighting the importance of innate immunity in defence against viral infections.

2. Materials and Methods

The open reading frame of chIFITM3 codon-optimized and chemically synthesized (GeneArt, Life Technologies, Germany) in-fusion with flag-tag and sub-cloned to an improved form of RCASBP(A)-ΔF1 using *ClaI* and *MluI* restriction sites. This restriction digestion excised the *src* gene and replaced it with chIFITM3 while maintaining the splice acceptor signals. This new vector was designed as RCASBP(A)-chIFITM3. The sequence integrity and orientation were confirmed by Sanger's sequencing. To rescue recombinant RCASBP(A) retroviruses, we followed methods described previously [9]. Briefly, DF-1 cells (ATCC ID: CRL-3586) were transfected with each of the plasmids using Lipofectamine 2000 (Life Technologies, Germany) in OptiMEM with a pre-determined and optimized ratio of 1:3. DMEM Media (Life Technologies, Germany) were changed 6 h post-transfection and replaced DMEM supplemented with 5% FCS and 1% Penicillin/Streptomycin for 48 h (Life Technologies, USA).

Expression of chIFITM3 was confirmed by staining the flag tag. Briefly, DF-1 cells grown on coverslips in 24-well plates were infected with retroviruses for 48 h. Cells were then fixed for 1 h using 4% paraformaldehyde and permeabilised using 0.1%

Triton-X100 (Thermo Scientific, UK) before incubation with primary antibodies raised against either flag tag (Thermo Scientific, UK). Afterward, cells were incubated with corresponding secondary antibodies (Fisher Scientific, UK) for 2 h at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Abcam, USA), and the images were taken using a Zeiss confocal laser-scanning microscope.

Speific pathogen free (SPF) eggs were acquired from a local supplier in co-operation with the Veterinary Serum and Vaccine Research Institute (VSVRI), Agriculture Research Centre (ARC), Egypt. Transgene-expressing chick embryos were generated by inoculation of 10^6 RCASBP(A)-chIFITM3 or empty RCASBP(A)-WT infected DF-1 cells into SPF chicken eggs (n=30/group) through the intra-yolk sac using 24G needles at day 2 post-embryonation (ED2). Eggs were fixed for 2 h post-inoculation before incubation at 37 °C with 60–80% humidity in a rotating incubator (twice daily). Embryos were allowed to hatch naturally at 21 days of incubation (ED21). Each group of transgene-expressing chicks was housed separately in containment level 3 isolators. Food and water were provided *ad libitum* and animal care was provided by the animal house staff.

The virus dosage optimization (clinical and sub-lethal doses) for H5N1 was carried out as outlined in our previouse study [10]. Experimentally, a total of 20 chicks expressing RCASBP(A)-chIFITM3, 20 chickens infected with RCASBP(A)-WT, and 15 mock-inoculated chicks (positive control) were challenged intranasally with 10^4 EID_{50} H5N1 (clinical dose) 12 days post-hatching. A total of 10 chicks were kept as a naïve negative control group (non-inoculated-non challenged, inoculated with PBS). All birds were monitored for the following 15 days to assess the appearance of clinical signs, weight gain, and mortalities. The experiment was terminated on day 35 and all

remaining chicks were euthanized. Quantification of influenza viruses was performed as we reported earlier [11].

Total RNA was extracted from tracheas (n=5/group) and lungs (n=5/group) using TRIzol reagents (Life Technologies, USA), which were collected from transgene-expressing (RCASBP(A)-chIFITM3) and mock chicks (mock-treated neg. ctrl). The RNA concentrations were measured using NanoDrop® ND-1000 UV-Vis Spectrophotometer and a total of 150 ng of RNA was used in the PCR reactions using SuperScript III Platinum SYBR Green One-Step qRT-PCR (Thermo Fisher Scientific) as described by the manufacturer. The abundance of specific chIFITM3 mRNA (qchIFITM3-F: 5'-ACTGTACGCCAATGTGTG-3', and qchIFITM3-R: 5'-TTGATCAGGTGAGCTGTG-3') was quantified and normalized with house keeping gene of 28S rRNA (qch28S-F: 5'-GGCGAAGCCAGAGGAAACT-3', and qch28S-R: 5'-GACGACCGATTTGCACGTC-3'). The reactions were run using a CFX96 Real-Time PCR machine and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method as we reported earlier (9, 10).

Oropharyngeal swabs were collected separately, placed in virus transport medium, filtered through a 0.2 um filter, and then aliquoted and stored at -70ºC until all samples were collected before analysis using hemagglutination assay and egg infective dose 50 (EID50) as we reported earlier (11). Selections of tissues including trachea, lung and spleen were collected and fixed at room temperature for 48 h by immersion in 10% neutral buffered formalin followed by paraffin wax embedding. The 5 µm tissue sections were stained using Hematoxylin and Eosin stain (Abcam, USA) before examination under a light microscope for microscopic lesions.

3. Results

In order to determine the *in vivo* antiviral activity of chIFITM3 against highly pathogenic avian influenza virus (HPAIV) subtype H5N1 (clade 2.2.1.2), chicks stably expressing chIFITM3 were generated. For this purpose, avian retroviruses were exploited *via* a vector-based expression system as we have reported earlier [11]. Specifically, the full-length open reading frame of chIFITM3 was cloned into RCASBP(A) vector between two unique restriction sites to efficiently express a caped and poly-adenylated chIFITM3 transcript (referred to as RCASBP(A)-chIFITM3) (**Fig. 1A**). RCASBP(A)-WT was used as a negative control in the over-expression experiment. The over-expression of chIFITM3 was confirmed in stably RCASBP(A)-chIFITM3 infected chicken embryo fibroblast (DF-1) cells using immunofluorescence assay (**Fig. 1B**). The expression of chIFITM3 was observed exclusively in the cytoplasm where the protein was detected in concentrated punctate (**Fig. 1B**). As expected, DF-1 cells stably infected with RCASBP(A)-chIFITM3 showed significant antiviral activities against HPAIV subtype H5N1 compared to wild type DF-1 cells (**Figure 1C**). These results confirm that RCASBP(A)-mediated expression of chIFITM3 is functionally stable and represents native antiviral function against HPAIV and these infectious cells can be exploited for overexpression of transgene (i.e. IFITM3) in developing chicks.

For the generation of chIFITM3-expressing chicks, 2-day-old embryonated eggs (ED2) were inoculated with recombinant RCAS virus (RCASBP(A)-chIFITM3 or RCASBP(A)-WT) infected DF-1 cells (**Fig. 2A**). The hatched chicks at ED21 were shifted to isolators in groups until challenged with clinical dose (10^4 EID_{50}) of HPAI H5N1 at 15 days post-hatching (PH15). Two independent experiments were performed to confirm that the expression of chIFITM3 had no detrimental effect on the chick's embryonic development and hatchability of RCASBP(A)-chIFITM3 inoculated eggs compared to the mock group (**Fig. 2B**). Furthermore, it was also noted that all RCASBP(A)-

chIFITM3 or RCASBP(A)-WT infected chicks carried a non-significant body weight reduction (**Fig. 2C**) albeit a subtle drop in weight gain in chIFITM3-overexpressing chicks soon after hatching. However, these chicks then progressively re-gained their body weight equal to the body weights of mock-inoculated group (negative control, inoculated with PBS) on the 15th day post-hatch. To confirm that chIFITM3 was expressed successfully in developing chicks, a transgene chIFITM3-specific quantitative RT-PCR was carried out. Owing to the expression of codon-optimized chIFITM3 through RCASBP(A), the PCR distinguished the transgene from endogenously expressed chIFITM3. Using this system, a significantly higher level of chIFITM3 was detected in tracheal RNA from chickens infected with RCASBP(A)-chIFITM3 compared to control groups (either transgene-expressing chicks, or non-transgene carrying-chicks) indicating the successful expression of chIFITM3 (**Fig. 2D**). Collectively, these findings highlight that RCASBP(A) virus causes no significant observable alterations in chicks and thus can reliability be challenged with HPIAV to demonstrate the antiviral actions of chIFITM3 *in vivo*.

There is a direct correlation between infectious virus dose and the severity of the clinical infections [12]. Additionally, the genetics of the H5N1 and host determine the clinical outcome of the infection. Therefore, it was critical to determine the inoculum titre of H5N1 virus that was able to induce clinical disease in chickens. Based on our previous studies [12], the pre-optimized dose of 10^4 EID_{50} (hereafter called clinical dose) of H5N1 strain A/chicken/Egypt_128s_2012 (clade 2.2.1.2) was used as a challenge virus to demonstrate the antiviral potential of chIFITM3 in chicks.

We observed full protection of chIFITM3-expressing chicks from clinical signs against challenge with the clinical dose of H5N1 virus. In contrast, H5N1-challenged chicks showed severe clinical signs from the 3rd day post-virus inoculation compared to

chicks in mock and non-challenged (negative control) which remained healthy. In addition, 60% of chicks expressing chIFITM3 were protected from clinical challenge without any apparent clinical disease (**Fig. 3A**). These results revealed that chicks over-expressing chIFITM3 show disease tolerance which resulted in delayed clinical signs by at least 7 days. Overall, the results show that the over-expression of chIFITM3 has a substantial impact on the outcome of the H5N1 infection (disease and mortality). Next, oropharyngeal swabs were collected from all groups (RCASBP(A)-chIFITM3, RCASBP(A)-WT, and mock-treated) before the challenge and after every alternative day post-clinical challenge to evaluate if chIFITM3 can mediate a reduction in virus shedding through oropharyngeal routes. The virus quantification results showed that chicks over-expressing chIFITM3 following clinical challenge showed a significant reduction in virus shedding (**Fig. 3B**) indicating that chIFITM3 is a key factor in virus replication that contributes to lowering influenza viral shedding.

Additionally, trachea, lung, and spleen organs were collected from chIFITM3-overexpressing challenged chicks, non-challenged, and non-inoculated (negative control) chicks at PH30 followed by histopathological examination. The mock negative control showed no observable histopathological lesions. Specifically, the trachea of chIFITM3-expressing chicks showed focal necrosis of lamina epithelialis (black arrow), congestion (red arrow) and inflammatory cells infiltration (blue arrow) (**Fig. 3C**). In contrast, the trachea of mock positive control showed edema in the lamina propria/sub-mucosal layer (arrow). Additionally, lungs of chIFITM3-expressing and mock positive control chicks showed reduced inflammatory cells infiltration in the air capillaries (black arrow) associated with interlobular edema (red arrow) compared to control lungs, indicating alleviation of pathological lesions in the respiratory tract. Correspondingly, spleen of chIFITM3-expressing and mock positive control showed

lymphocytic necrosis and depletion (arrow) compared to negative control. Collectively, the histopathological observations indicate that over-expression of chIFITM3 reduced the H5N1-induced pathology in studied organs which resulted in reduced clinical signs in chicks.

4. Discussion

Virus-mediated innate immune responses and mechanistic observations vary between avian and mammals, and these cross-species host restriction factors may determine the steps involved in virus pathogenesis. The first line of defense against viral infection is the activation of the innate immune response in almost all vertebrate cells [13, 14]. IFITM genes are one of the highly upregulated ISGs and are found in a wide variety of animals including mammals, fish, birds, and amphibians. The chIFITM1 and chIFITM3 have been genetically and functionally characterized [15] and although most studies provide data in *in vitro* only, the results provide key antiviral functions and suggest avenues for further investigations.

The IFITMs are widely expressed IFN-inducible proteins that prevent infection by several viruses such as influenza A virus (IAV), West Nile virus (WNV), dengue virus (DENV), severe acute respiratory syndrome coronavirus (SARS-CoV), vesicular stomatitis virus (VSV) and hepatitis C virus (HCV) [4]. According to these studies Ifitm3$^{-/-}$ mice had increased vulnerability to respiratory syncytial virus [16], West Nile virus [17], arthritogenic and encephalitic alphaviruses [18], and influenza [19]. Although previous research suggested a connection between IFITM3 and the control of antiviral immunity, the direct effect of IFITM3 on viral replication has not been clearly separated from any of IFITM3's immune-regulatory roles. Experimental data have

indicated that enhanced viral pathogenicity in hosts with weak or impaired IFITM3 activity is a result of impaired restriction of virus entry and replication [4].

In order to determine if chIFITM3 affects the pathogenesis of the influenza virus *in vivo*, we applied an over-expression approach. While different approaches to generate transgenic chicken have been applied for studying microbial pathogenesis and chicken physiology (22), RCAS vector system offers a simple and convenient tool to study the functions of cellular genes (11). We found that the checkpoint regulator chIFITM3 is a crucial component of influenza-induced immunological dysfunction during *in vivo* infection using the RCAS retrovirus gene transfer system. The chIFITM3 functions as a rheostat of antiviral immunity that controls the pathogenic outcome of influenza virus infection since chIFITM3 doesn't entirely prevent influenza virus replication. Our results showed a non-significant body weight loss in RCAS-mediated transgene-expressing chicks after hatching. In comparison, hatched chicks regained weight efficiently and obtained comparable sizes to non-transgene expressing chicks. In this study, chIFITM3-overexpressing chicks were established to further explore the *in vivo* antiviral function of chIFITM3 against HPAIV subtype H5N1. Our results provide strong evidence that chIFITM3 can significantly protect transgene-expressing chicks (~60%) against the clinical dose of the H5N1 avian influenza virus that causes clinical disease. Due to variable conditions that can influence poultry susceptibility to infections (20-21), further investigation was carried out to understand the impact of chIFITM3 against a pre-determined clinical dose of H5N1. The results revealed that the clinical dose of H5N1 considerably reduced the clinical outcome when used in transgene-overexpressing chicks, which indicates the ability of innate immunity to protect against HPAIV H5N1. We have previously applied a similar approach to investigate the impact of chIFITM1 against the influenza virus and noticed a higher and profound inhibition

of influenza-induced pathology in chicken (11) compared to chIFITM3 reported in this study. Collectively, our findings revealed that over-expression of chIFITM3 or chIFITM1 reduces not only the clinical disease in H5N1-infected chicks but also virus-induced pathological lesions and virus shedding.

To conclude, findings indicate the importance of the innate immune system in establishing an antiviral state against HPAIV. The presented data provide proof of the capacity to generate virus-resistant chickens which can protect food and inhibit the long-term spread of zoonotic viruses to humans. Gaining a further understanding of factors that influence the susceptibility of poultry to avian influenza viruses will help to reduce the risks to animal and human health.

Ethical clearance

The study was approved by the Institutional Ethical Committees of Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Egypt.

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Declaration of competing interest

Authors declare no competing interest.

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Figure captions:

Fig. 1. Construction and confirmation of RCASBP(A) expression system for chIFITM3. (A) schema for the generation of recombinant RCASBP viruses in which *src* gene was replaced with chIFITM3. (B) Confirmation of RCASBP(A)-mediated stable expression of chIFITM3 using immunofluorescence assay. Stably-infected cells expressing chIFITM3 were fixed, permeabilized and stained using anti-flag antibodies targeting the N-terminal flag fused with the chIFITM3 (C) Stable cells expressing chIFITM3 or wild type cells were infected with HPAIV for 24 hours with an moi of 1.0. Cell supernatant was used to quantify the secreted virus using plague assay. The data represent experiments conducted in triplicate. **** indicates the level of significance at p value < 0.0001.

Fig. 2. Experimental layout for transgene-overexpression and impact of chIFITM3 on hatchability and weight gain of hatched chicks. (A) Experimental layout to generate chicks expressing chFITM3. ED represents embryonation day and PH represents post hatching days. (B) Comparison of hatchability percentage for eggs post-chIFITM3 or mock inoculation. (C) Average body weight gain in percentage in chicks post hatching compared to mock chicks. (D) Expression of chIFITM3 in RCASBP(A)-chIFITM3 infected and H5N1 challenged chicks compared to chicks infected with (RASBP(A)-WT) and mock inoculated (neg. ctrl). Asterisks (****) indicates level of significant differences (p=0.0001).

Fig. 3. Clinical outcome of transgene-expressing chicks compared to wild type chicks challenged with HPAIV. (A) Percentage survival rates of RCASBP(A)-chIFITM3 and RCASBP(A)-WT infected and challenged chicks with clinical doses of H5N1 compared to mock inoculated chicks (negative and positive control groups).

(B) Evaluation of viral shedding from oropharyngeal swabs of virus challenged and RCASBP(A)-chIFITM3 and RCASBP(A)-WT infected chicks compared to mock inoculated chicks (negative and positive control groups). (C) Photomicrographs representing H&E-stained sections of tracheas, lungs and spleen collected from RCASBP(A)-chIFITM3 infected and H5N1 challenged chicks compared to mock inoculated chicks (negative and positive groups) at post hatching day 30. (scale bar = 25um). **** indicates the level of significance at p value < 0.0001.