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RESEARCH ARTICLE

Development of a chicken interferon-induced transmembrane protein 3 (IFITM3)-specific monoclonal antibody using phage display

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ABSTRACT

Interferon-induced transmembrane protein 3 (IFITM3) has potent antiviral activity against several viruses. Recent studies have reported that the chicken *IFITM3* gene also plays a pivotal role in blocking viral replication, but these studies are considerably limited due to being conducted at the RNA level only. Thus, the development of a chicken IFITM3 protein-specific antibody is needed to validate the function of IFITM3 at the protein level. Epitope prediction was performed with the immune epitope database analysis resource (IEDB-AR) program. The epitope was validated by four *in silico* programs, Jped4, Clustal Omega, TMpred and SOSUI. Chicken IFITM3 protein-specific monoclonal antibodies were screened by enzyme-linked immunosorbent assay through affinity between recombinant IFITM3 protein and phage-displayed candidate antibodies. Validation of the reactivity of the chicken IFITM3 protein-specific antibody to chicken tissues was carried out using western blotting. We developed a chicken IFITM3 protein-specific monoclonal antibody using phage display. The reactivity of the antibody with peripheral chicken tissues was confirmed using western blotting. To the best of our knowledge, this was the first development of a chicken IFITM3 protein-specific monoclonal antibody using phage display.

KEYWORDS

IFITM3, antibody, chicken, phage display

INTRODUCTION

Interferon-induced transmembrane protein 3 (IFITM3) encoded by the *IFITM3* gene is a potent antiviral effector modulated by interferon against a wide range of viruses, including Ebola virus, Marburg virus, severe acute respiratory syndrome coronavirus, dengue virus, West Nile virus, Zika virus and influenza A viruses (Brass et al., 2009; Everitt et al., 2012; Diamond and Farzan, 2013; Gorman et al., 2016; Allen et al., 2017; Kim and Jeong, 2017; Kim et al., 2020, Kim and Jeong, 2021). The IFITM3 protein is a member of the CD225 superfamily, and this domain is well conserved in the mammalian IFITM3 proteins. The IFITM3 protein plays a crucial role in blocking viral hemifusion by composing a chain-like octamer structure on the host cellular membrane (Feeley et al., 2011; Spence et al., 2019; Kim and Jeong, 2021). In addition, the IFITM3 protein has been found to be expressed in CD4+ T cells and to regulate T cell differentiation via the T cell receptor signalling complex (Yanez et al., 2019, 2020). These studies indicate that the IFITM3 protein is a major component of the innate and adaptive immune systems.

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In birds, the IFITM3 protein is also conserved in several avian species, including chickens, ducks and geese (Smith et al., 2015a, b; Blyth et al., 2016; Wang et al., 2017; Bassano et al., 2019; Kim et al., 2019). In addition, avian reovirus and infectious bursal disease virus induce dramatically elevated expression of the avian *IFITM3* gene (Smith et al., 2015a, b; Wang et al., 2021). Furthermore, the chicken *IFITM3* gene has been significantly overexpressed against avian Tembusu virus, influenza viruses and lyssaviruses, and has been shown to restrict replication of those viruses (Smith et al., 2013; Chen et al., 2017). However, previous studies on the chicken *IFITM3* gene were carried out at the RNA level only and have not been confirmed at the protein level thus far (Smith et al., 2015b; Wang et al., 2021). Since the chicken IFITM3 protein shows low sequence identity with mammalian IFITM3 proteins, the reactivity of mammalian IFITM3 protein-specific antibodies is not applicable in chickens (Kim et al., 2019). Thus, the development of chicken-IFITM3-specific antibodies is highly desirable.

To develop a monoclonal antibody against the chicken IFITM3 protein, we performed epitope prediction with the immune epitope database analysis resource (IEDB-AR) program and validated the epitope by four *in silico* programs, Jped4, Clustal Omega, TMpred and SOSUI (Cuff et al., 1998; Hirokawa et al., 1998; Sievers and Higgins, 2014). In addition, we performed phage display and screened it for antibodies to chicken IFITM3 using an enzyme-linked immunosorbent assay (ELISA). Furthermore, we evaluated the reactivity of the specific antibody to several chicken tissues by western blot analysis.

MATERIALS AND METHODS

Experimental birds

A total of 3 Korean native chickens (3 months old) were provided to obtain chicken tissues for western blot analysis from an animal farm in the Republic of Korea. All experimental protocols were approved by the Institutional of Animal Care and Use Committee (IACUC) of Jeonbuk National University (JBNU 2017–0030).

Information on the amino acid sequences of the IFITM1 and 3 proteins

The amino acid sequences of the chicken IFITM3 (GenBank ID: NP_001336990.1) and IFITM1 (GenBank ID: NP_001336988.1) proteins were obtained from GenBank at the National Center for Biotechnology Information (NCBI).

Epitope prediction and secondary structure analysis

The epitope of the chicken IFITM3 protein was predicted by the Immune Epitope Database and Analysis Resource (IEDB-AR) (https://tools.iedb.org/bcell/) with Bepipred Linear Epitope Prediction 2.0 method (aa 4–43). The epitope '^{12}PPYEPLMDGMDMEGKTRS^{29}' was selected according to high epitope score (>0.6) calculated from the epitope database.

The secondary structure of the chicken IFITM3 protein with 137 amino acids was predicted by the Jped4 program (https://www.compbio.dundee.ac.uk/jpred/). The transmembrane domain of the chicken IFITM3 protein was predicted by TMpred (https://www.ch.embnet.org/software/TMPRED_form.html) and SOSUI (https://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html).

Pairwise sequence alignment was performed for two amino acid sequences, those of the chicken IFITM1 and 3 proteins, using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Synthesis of epitope peptide of chicken IFITM3 protein and recombinant chicken IFITM3 protein (rIFITM3)

The epitope peptide of chicken IFITM3 protein, 'PPYEPLMDGMDMEGKTRS', which was derived from the full-length chicken IFITM3 protein, was used as an immunogen in this study. The epitope peptide of chicken IFITM3 protein was synthesised by ANYGEN (Gwangju, Republic of Korea).

The full length of rIFITM3 was used for positive control of western blotting in this study. rIFITM3 was synthesised by BIONEER (Daejeon, Republic of Korea).

Development of phage display-derived chicken IFITM3 protein-specific antibodies

The scFv clones originated from a chicken naïve phage library (YntoAb, Seongnam, Republic of Korea) that was screened by three rounds of biopanning using the synthesised epitope peptide followed a previous study (Kim et al., 2021a). Monoclonal binders were selected using an ELISA based on the synthesised epitope peptide. Chicken IFITM3 protein-specific scFv binders were inserted into scFv-Fc vectors and expressed in *E. coli* TG1 (YntoAb, Seongnam, Republic of Korea). Expression of the monoclonal scFv-Fc antibody was induced by the addition of isopropyl β-D-1-thiogalactopyronoside (IPTG) (Sigma-Aldrich, Missouri, USA) to the bacterial culture. Bacterial cells were lysed by sonicator (Thermo Fisher Scientific, MA, USA), and the monoclonal scFv-Fc antibody was purified using Ni^{2+}-affinity chromatography (HisTrap FF; GE Healthcare, IL, USA). After dialysis with phosphate buffered saline (PBS) (Bioneer, Daejeon, Republic of Korea), the antibody concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA).

ELISA

A 96-well plate was coated with each rIFITM3 antigen (100 ng) for 1 h at 37 °C and then blocked with 5% bovine serum albumin (BSA). Each scFv-Fc antibody (1:100 dilution) was added to a blocked well, and the sample was incubated for 1 h at room temperature. After washing with Tris-buffered saline with 0.1% Tween 20 (TBST), a horseradish peroxidase-conjugated anti-HA antibody (1:2,000; Thermo Fisher Scientific, MA, USA) was added, and the sample was

incubated for 1 h at room temperature. After washing with TBST, binding affinity was estimated by addition of an ELISA substrate (1-Step Ultra TMB-ELISA; Thermo Fisher Scientific), and colorimetric signals were detected using a Synergy HTX plate reader (BioTek Instruments, Winooski, VT).

Western blotting

A total of 4 peripheral tissues, the bronchus, bursa of Fabricius, caecum and rectum, were collected and homogenised with a 10% volume of RIPA lysis buffer (Thermo Fisher Scientific, Waltham, USA) containing a protease inhibitor cocktail (Roche, Munich, Germany). Equal amounts of protein (80 µg) were heated to 95 °C for 10 min in 5X sample buffer (Thermo Fisher Scientific, Waltham, USA) and loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis at 100 V for 1.5 h, the separated proteins were transferred to an AmershamTM nitrocellulose membrane by a trans blot apparatus (BioRad, Hercules, USA). The membranes were washed with TBST and then blocked in TBST containing 5% skim milk (Santa Cruz Biotechnology, Dallas, USA) for 2 h. The membranes were then incubated for 6 h at room temperature with the anti-chicken IFITM3 monoclonal antibody. After washing in TBST, the membranes were incubated with a horseradish peroxidase-conjugated anti-HA antibody (1:2,000; Thermo Fisher Scientific, MA, USA) for 1 h and washed in TBST again. The targeted proteins were visualised with a Pierce ECL kit (Thermo Fisher Scientific, Waltham, USA).

RESULTS

Epitope selection

The IEDB-AR program, run on the 137-amino-acid long chicken IFITM3 protein (GenBank ID: NP_001336990), predicted the following peptide sequence PPYEPLMDGMDMEGKTRS (in aa positions 12–29) as an epitope (Fig. 1A). Since the coil structure is preferred to the β-sheet structure as an epitope, we performed secondary structure analysis of the chicken IFITM3 protein and identified this oligopeptide stretch at a coil-rich structure (Fig. 1A).

A previous study (Bassano et al., 2019) has reported high homology between the chicken IFITM1 and IFITM3 proteins. To avoid cross-reactivity, we performed pairwise sequence alignment among the amino acid sequences of the chicken IFITM1 and 3 proteins and confirmed that the epitope avoided cross-reactivity (Fig. 1B). Since transmembrane domains as epitopes show low reactivity with monoclonal antibodies, we performed transmembrane domain analysis and confirmed that the epitope peptide sequence was not located in the transmembrane domain and was located in the N-terminal outer membrane domain (Fig. 1C). We also investigated whether the epitope was changed by genetic polymorphisms. All genetic polymorphisms, including L100M, V103I and N125H, of the chicken *IFITM3* gene identified in a previous study (Kim et al., 2019) were not located on the epitope located at residues 12–29 of the chicken IFITM3 protein (Fig. 1C).

Development of the chicken IFITM3 protein-specific monoclonal antibody

We carried out phage display to select the chicken IFITM3 protein-specific monoclonal antibody using the epitope peptide and chicken naïve phage library (YntoAb, Seongnam, Republic of Korea) (Kim et al., 2021a). After three rounds of biopanning and ELISA screening, 10 specific primary clones for epitope peptide of the chicken IFITM3 protein were selected. To generate scFv-Fc fusion proteins, the sequences of the selected clones were confirmed, and scFv-Fc fusion proteins were produced based on this sequence information. Finally, chicken IFITM3 protein-specific scFv-Fc fusion proteins were screened by western blotting (data not shown). Among them, the 2E1 clone originating from the scFv-Fc fusion proteins showed reactivity to rIFITM3 and not to BSA (negative control) (Fig. 2A).

To evaluate the reactivity of the chicken IFITM3 protein-specific antibody to chicken tissues, we performed western blotting in 4 peripheral tissues, the bronchus, bursa of Fabricius, caecum and rectum. The chicken IFITM3 protein-specific antibody showed specific reactivity in all chicken tissues tested (Fig. 2B).

DISCUSSION

A previous study reported that the IFITM3 protein of chickens showed a considerable difference in amino acid sequence from that of the IFITM3 protein of mammals. In addition, because of the significant difference in the amino acid sequence of the chicken IFITM3 protein, the topology of the chicken IFITM3 protein was also different compared to that of the chicken IFITM3 protein of mammals. The N-terminal and C-terminal domains of the avian IFITM3 protein are longer than those of the mammalian IFITM3 protein. Furthermore, the promoter structure of the chicken *IFITM3* gene is also different from the mammalian *IFITM3* gene. Chickens but not mammals contain CpG islands in the promoter region of the *IFITM3* gene (Kim et al., 2019). Since several differences in the *IFITM3* genes have been observed between mammals and chickens, there may also be differences in the IFITM3 protein between mammals and chickens. Thus, it is highly desirable to perform future studies at the protein level on the chicken IFITM3 protein using the antibody developed in this study.

In previous studies, the rs12252 single nucleotide polymorphism (SNP) of the human *IFITM3* gene, which is located on the splice acceptor site, has been found to be associated with a splicing variant of the *IFITM3* gene and related to the severity of pandemic influenza H1N1 2009

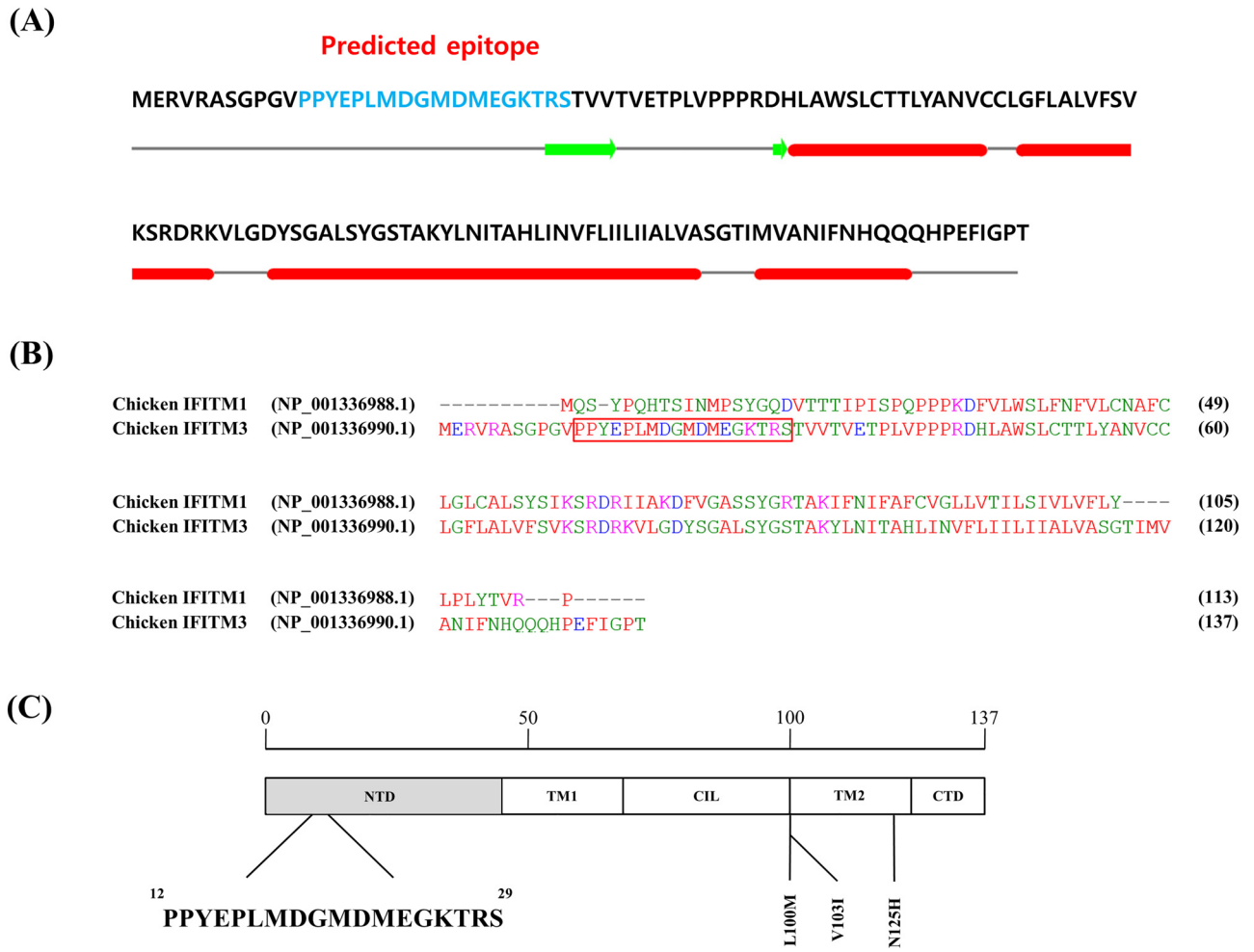

Fig. 1. In silico evaluation of the epitope of chicken interferon-induced transmembrane protein 3 (IFITM3). (A) Secondary structure of the IFITM3 protein and the location of the predicted epitope. The red and green arrows indicate α-helices and β-sheets, respectively. The grey lines indicate coils. (B) Pairwise sequence alignments between the chicken IFITM3 and IFITM1 proteins and the location of the predicted epitope. The red box indicates the predicted epitope. (C) Topology of the chicken IFITM3 protein and the location of genetic polymorphisms of the *IFITM3* gene. NTD: N-terminal domain; TM1: transmembrane 1; CIL: conserved intracellular loop; TM2: transmembrane 2; CTD: C-terminal domain

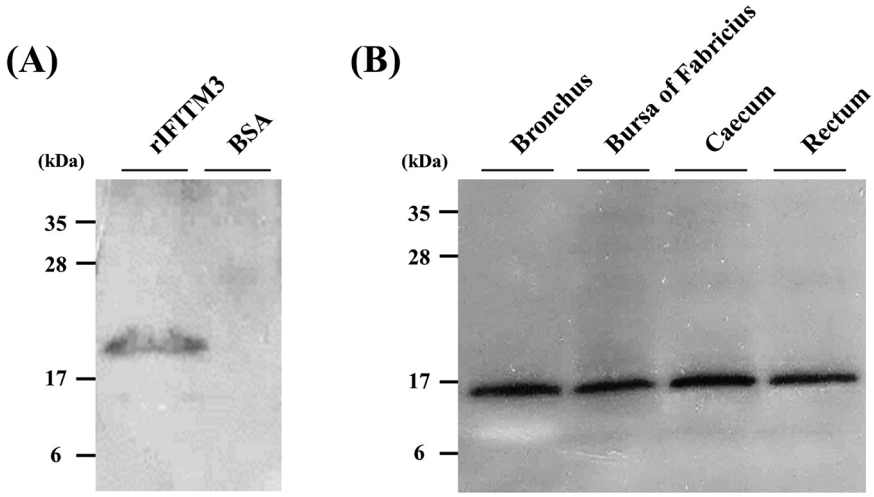

Fig. 2. Western blotting results using the chicken IFITM3 protein-specific monoclonal antibody. (A) Western blotting results of the recombinant IFITM3 protein (rIFITM3, positive control) and bovine serum albumin (BSA, negative control). (B) Western blotting results of chicken peripheral tissues

infection in East Asian and Caucasian populations (Everitt et al., 2012; Zhang et al., 2013; Chen et al., 2018). However, this polymorphism was not associated with susceptibility to pandemic influenza H1N1 2009 infection in the Korean population (Kim and Jeong, 2017; Kim et al., 2021b). The rs34481144 SNP, which is located on the binding region of CCCTC-Binding Factor (CTCF), affects the severity of pandemic influenza H1N1 2009 infection (Allen et al., 2017). In addition, the rs6598045 SNP, which is located on the binding region of TFII-I, plays a pivotal role in susceptibility to pandemic influenza H1N1 2009 (Kim et al., 2020). Recently, *IFITM3* SNPs also showed an association with the severity of COVID-19 in several ethnic groups (Kim and Jeong, 2020; Gomez et al., 2021; Schonfelder et al., 2021). In chickens, three nonsynonymous SNPs, L100M, V103I and N125H, have been reported with high frequencies. According to the haplotype of these three nonsynonymous SNPs, alteration of the position of the transmembrane domain has been observed (Kim et al., 2019). Since the topology of the IFITM3 protein is important to inhibit viral invasion and nonsynonymous SNPs affect the topology of the chicken IFITM3 protein (Bailey et al., 2013), further analysis of the association between the nonsynonymous SNPs of the chicken *IFITM3* gene and susceptibility to several avian viruses will become possible in the future.

In the present study, we tested only the reactivity of the chicken IFITM3 protein-specific monoclonal antibody using ELISA and western blotting. In a previous study, the amino acid sequence of the chicken IFITM1 protein was found to have high homology with that of the chicken IFITM3 protein, and it was difficult to name the IFITM1 and 3 proteins. In mammals, the IFITM3 and 1 proteins are mainly localised in the late endosome and on the cell surface, respectively. In addition, the roles of the IFITM3 and IFITM1 proteins in the prevention of viral invasion are different (Brass et al., 2009; Li et al., 2013; Smith et al., 2014; Yanez et al., 2020). Since we developed a chicken IFITM3-specific monoclonal antibody in the present study, further investigation of the antiviral capacity of the chicken IFITM3 protein according to the cellular compartment is highly desirable. For this, further validation of the reactivity of the chicken IFITM3 protein-specific monoclonal antibody using immunocytochemistry and immunohistochemistry is warranted. In addition, further investigation of the reactivity of the chicken IFITM3 protein-specific monoclonal antibody in other avian species is needed in the future.

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