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Molecular characterization and expression of a disintegrin and metalloproteinase with thrombospondin motifs 8 in chicken

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Objective: A disintegrin and metallopeptidase with thrombospondin motifs type 8 (ADAMTS8) is crucial for diverse physiological processes, such as inflammation, tissue morphogenesis, and tumorigenesis. The chicken *ADAMTS8* (*chADAMTS8*) gene was differentially expressed in the kidney following exposure to different calcium concentrations, suggesting a pathological role of this protein in metabolic diseases. We aimed to examine the molecular characteristics of chADAMTS8 and analyze the gene-expression differences in response to toll-like receptor 3 (TLR3) stimulation.
Methods: The *ADAMTS8* mRNA and amino acid sequences of various species (chicken, duck, cow, mouse, rat, human, chimpanzee, pig, and horse) were retrieved from the Ensembl database and subjected to bioinformatics analyses. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) experiments were performed with various chicken tissues and the chicken fibroblast DF-1 cell line, which was stimulated with polyinosinic-polycytidylic acid (poly[I:C]; a TLR3 ligand).
Results: The *chADAMTS8* gene was predicted to contain three thrombospondin type 1 (TSP1) domains, whose amino acid sequences shared homology among the different species, whereas sequences outside the TSP1 domains (especially the amino-terminal region) were very different. Phylogenetic analysis revealed that chADAMTS8 is evolutionarily clustered in the same clade with that of the duck. *chADAMTS8* mRNA was broadly expressed in chicken tissues, and the expression was significantly up-regulated in the DF-1 cells in response to poly(I:C) stimulation ($p<0.05$). These results showed that *chADAMTS8* may be a target gene for TLR3 signaling.
Conclusion: In this report, the genetic information of *chADAMTS8* gene, its expression in chicken tissues, and chicken DF-1 cells under the stimulation of TLR3 were shown. The result suggests that *chADAMTS8* expression may be induced by viral infection and correlated with TLR3-mediated signaling pathway. Further study of the function of chADAMTS8 during TLR3-dependent inflammation (which represents RNA viral infection) is needed and it will also be important to examine the molecular mechanisms during different regulation, depending on innate immune receptor activation.

Keywords: Chicken; ADAMTS8; Protein Structure; Gene Expression; Innate Immune Receptor Signaling

INTRODUCTION

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) are secreted, extracellular enzymes with a compound domain organization [1]. In humans, nineteen ADAMTS proteins are encoded in the genome and they can be clustered into eight 'clades' based on their domain organization and known functions. The aggrecanase and proteoglycanase clades (ADAMTS1, 4, 5, 8, 9, 15, and 20) can cleave hyaluronan-binding chondroitin sulfate proteoglycans, a type of extracellular proteins, including aggrecan, versican, brevican, and neurocan [2]. Among them, ADAMTS8, also known as metalloprotease and thrombo-

spondin domains 2 (METH-2), is a member of the ADAMTS family and was originally identified as an antiangiogenic factors that is down-regulated in brain cancer [3,4]. *ADAMTS8* down-regulation has been also reported in other cancers, including brain, breast, lung, pancreatic, and thyroid cancer [4-7]. The *ADAMTS8* gene shows a high frequency of promoter methylation in brain, lung, and thyroid cancer, suggesting that the epigenetic silencing of *ADAMTS8* may be involved in tumorigenesis [4,6,8]. Moreover, in cancer cells regulated by ADAMTS8, certain mutations can drive abnormal signaling during viral infection, indicating a correlation between cancers and viruses [9,10].

Pathogen-associated molecular patterns (PAMPs), such as polyinosinic-polycytidylic acid (poly[I:C]) and lipopolysaccharide, have been used to study toll-like receptor (TLR)-mediated cellular responses. Poly(I:C) is a synthetic analogue of double-stranded RNA (dsRNA), a PAMP generated during the replication of RNA and DNA viruses [11], and is recognized by distinct receptors depending on their localization. When added to the culture medium, poly(I:C) is mainly sensed by endosome-localized TLR3 [12,13]. TLR3 signaling can also occur in non-immune cells, contributing to an antitumor response. TLR3 is activated by extracellular dsRNA, which is recognized by the receptor in a sequence-independent manner. TLR3 initiates a protective response against dsRNA viruses including polio virus, coxsackievirus group B and serotype 3, and encephalomyocarditis virus, as well as DNA viruses, such as herpes simplex virus 1 and murine cytomegalovirus [14-16]. Besides the mammals, TLR3 recognizes dsRNA virus infection and is involved in the resistance or susceptibility to viral infection in fowls. It was reported that the mRNA-expression levels of duck TLR3 and other cytokines (including interferon-α [IFN-α]) were highly up-regulated during infection by duck reovirus, a dsRNA virus [17]. In 2014, Cheng et al [18] reported that infection with the chicken Newcastle disease virus increased the mRNA expression of chicken TLR3. ChTLR3 actively participates in the recognition of pro-inflammatory responses during viral infection, and leads to consequent antiviral cytokine secretion in chickens. In addition, TLR3 recognizes dsRNA that has been transcribed *in vitro* and its synthetic analogues, such as poly(I:C) and polyadenylic:polyuridylic acid (poly [A:U]). These analogues have been used to mimic responses to RNA virus infection and are commonly administered in *in vitro* and *in vivo* studies of TLR3-mediated cellular responses [19].

Although the expression of chicken *ADAMTS8* (*chADAMTS8*) appears to be related to virus infection, the structure and expression pattern of this gene have not been studied. Here, we analyzed the amino acid sequence encoded by the *chADAMTS8* gene by comparing it with the ADAMTS8 amino acid sequences from other species. Gene expression was investigated in various chicken tissues. In addition, the expression pattern of the *chADAMTS8* gene was evaluated in the chicken DF-1 fibroblast cell line after stimulation with poly(I:C).

MATERIALS AND METHODS

Cell culture and stimulation

The chicken DF-1 cell line was purchased from the American Tissue Culture Collection (CRL-12203, Manassas, VA, USA). DF-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL each of penicillin and streptomycin (Thermo Scientific, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Poly(I:C) was purchased from Invivogen (San Diego, CA, USA) and used for stimulation at a concentration of 10 μg/mL for 24 h.

Bioinformatic analysis

The *ADAMTS8* mRNA and amino acid sequences of various species (chicken, duck, human, chimpanzee, mouse, rat, cow, pig, and horse) were retrieved from the Ensembl database (http://www.ensembl.org/) (Table 1) and aligned with the BioEdit software, using the ClustalW method. The protein domains were predicted by using the SMART domain search program (http://smart.embl-heidelberg.de/). Phylogenetic analyses were performed with the MEGA7 software [20].

Table 1. Ensembl and amino acid sequence IDs of *ADAMTS8* genes of various species

Species	Scientific name	Ensembl ID	NCBI Reference Sequence ID
Chicken	Gallus gallus	ENSGALG00000001370	XM_015298130.1
Duck	Anas platyrhynchos	ENSAPLG00000006051	XM_021272506.1
Human	Homo sapiens	ENSG00000134917	NM_007037.5
Chimpanzee	Pan troglodytes	ENSPTRG00000004483	XM_522252.4
Mouse	Mus musculus	ENSMUSG00000031994	NM_013906.3
Rat	Rattus norvegicus	ENSRNOG00000005574	XM_003750455.4
Cow	Bos taurus	ENSBTAG00000010411	NM_001192325.1
Pig	Sus scrofa	ENSSSCG00000015249	XM_003130083.4
Horse	Equus caballus	ENSECAG00000014164	XM_005611722.2

ADAMTS8, a disintegrin and metalloproteinase with thrombospondin motifs 8; NCBI, National Center for Biotechnology Information.

Reverse transcription-polymerase chain reaction

Trizol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissue samples (liver, lung, kidney, spleen, and heart) and DF-1 cells. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The *chADAMTS8*, *chTLR3*, and *chTLR4* transcripts were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) amplification. The RT-PCR conditions were as follows: an initial step of 94°C for 10 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final step of 72°C for 10 min. The RT-PCR products were analyzed by electrophoresis on a 2.0% SeaKem LE agarose gel (Lonza, Basel, Switzerland). Target gene expression was normalized against that of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. The sequences of the *GAPDH* primers were 5′-TGC TGC CCA GAA CAT CAT CC-3′ for the forward primer and 5′-ACG GCA GGT CAG GTC AAC AA-3′ for the reverse primer.

Quantitative real-time polymerase chain reaction analysis

Quantitative RT-PCR (qRT-PCR) was conducted with a CFX-96 RT-PCR detection system (BioRad, Hercules, CA, USA) to analyze *chADAMTS8*, *chTLR3*, *chTLR4*, and *chIL1B* expression. The sequences of the *chADAMTS8* primers were 5′-GCA CTA TGA CAC TGC CAT CCT-3′ for the forward primer and 5′-CGT GTC GCA GCC TTG ATG-3′ for the reverse primer. *ChTLR3* primers were: 5′-CCA TTT TGA AGG GTG GAG AA-3′ (forward) and 5′-CCT GCT TCG AAG TCT CGT TC-3′ (reverse). *chTLR4* primers were: 5′-TTC CAA GCA CCA GAT AGC AAC ATC-3′ (forward) and 5′-ACG GGT CAC AGA AGA ACT TAG GG-3′ (reverse). *chIL1B* primers were: 5′-GGA TTC TGA GCA CAC CAC AGT-3′ (forward) and 5′-TCT GGT TGA TGT CGA AGA TGT C-3′ (reverse). The PCR conditions were as follows: an initial step of 94°C for 3 min; 39 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s; and a final step of 72°C for 10 min. Dissociation was performed at 0.5°C increments from 55°C to 95°C over 25 min. All samples were measured in triplicate to ensure reproducibility, and Ct values were calculated by the $2^{-\Delta\Delta Ct}$ method [21]. Expression of the *GAPDH* gene was detected as the reference.

Statistical analysis

Results are presented as the means±standard deviation of triplicate independent experiments. Statistical significance was assessed using a Student's *t*-test. A p value of <0.05, compared with the non-treated control, was considered to reflect a statistically significant difference.

RESULTS AND DISCUSSION

The amino acid sequence encoded by *chADAMTS8* was analyzed and compared with that of the other species. *ChADAMTS8* was identified as a differentially expressed gene (DEG) from chicken kidney by RNA-Seq analysis, after the chickens were fed a diet containing different amounts of calcium [22]. The *chADAMTS8* amino acid sequence was compared with the duck, horse, pig, cow, mouse, rat, chimpanzee, and human sequences, which were retrieved from the Ensembl database. ChADAMTS8 was predicted to contain three thrombospondin type 1 (TSP1) domains, whose amino acid sequences were conserved among the species examined, whereas sequences outside of these domains (especially in the amino-terminal region) were diverse (Figure 1A). Specifically, all three TSP1 domains of duck ADAMTS8 shared homology with chADAMTS8, whereas only two TSP1 domains were homologous with those of the other mammal species. Nonetheless, this result suggests that the chADAMTS8 may serve similar biological functions as ADAMTS8 in other species, including antiangiogenic activity and tumorigenesis. The phylogenetic tree of ADAMTS8 revealed that chADAMTS8 is evolutionarily clustered in the same clade with that of the duck (Figure 1B).

ADAMTS8 is known to be related to the development of lung cancer and recently, Zhou et al [23] reported that ADAMTS8 can be used as a biomarker for human lung cancer [6]. The gene-expression pattern of *chADAMTS8* was investigated in various chicken tissues. RT-PCR and qRT-PCR analysis revealed that *chADAMTS8* gene was highly expressed in the chicken lung tissue under normal conditions (Figure 2A, 2B). In addition to lung tissue, *chADAMTS8* was also expressed in other tissues including spleen, kidney, heart, and liver. Like human *ADAMTS8* gene expression in normal adult and fetal lung tissues, *chADAMTS8* showed the highest expression in lung tissue and the second-highest expression in the spleen tissue. So far, it is not clear what the physiological role of chADAMTS8 is even though its expression is the highest in lung, and further study warrants revealing its role in normal condition as well as inflammatory conditions, caused by viral pathogens.

In addition, to gain insight into the regulation of *chADAMTS8* gene expression, we examined its expression in chicken DF1 cells that were stimulated with poly(I:C). To confirm the innate immune responses of chicken DF-1 cell is stimulated by poly(I:C), the activation of TLR3 and TLR4 was examined with the agonist treatment. As shown in Figure 3A, the expressional levels of TLR3 and TLR4 in chicken DF-1 cell were increased under stimulation of poly(I:C). Additionally, the downstream activation of TLR3 pathway in response to the poly(I:C) stimulation was observed with interleukin 1 beta expression which is a cytokine induced following TLR3 activation (Figure 3B). A recent study reported that DF-1 cells express higher level of suppressor of cytokine signaling 1

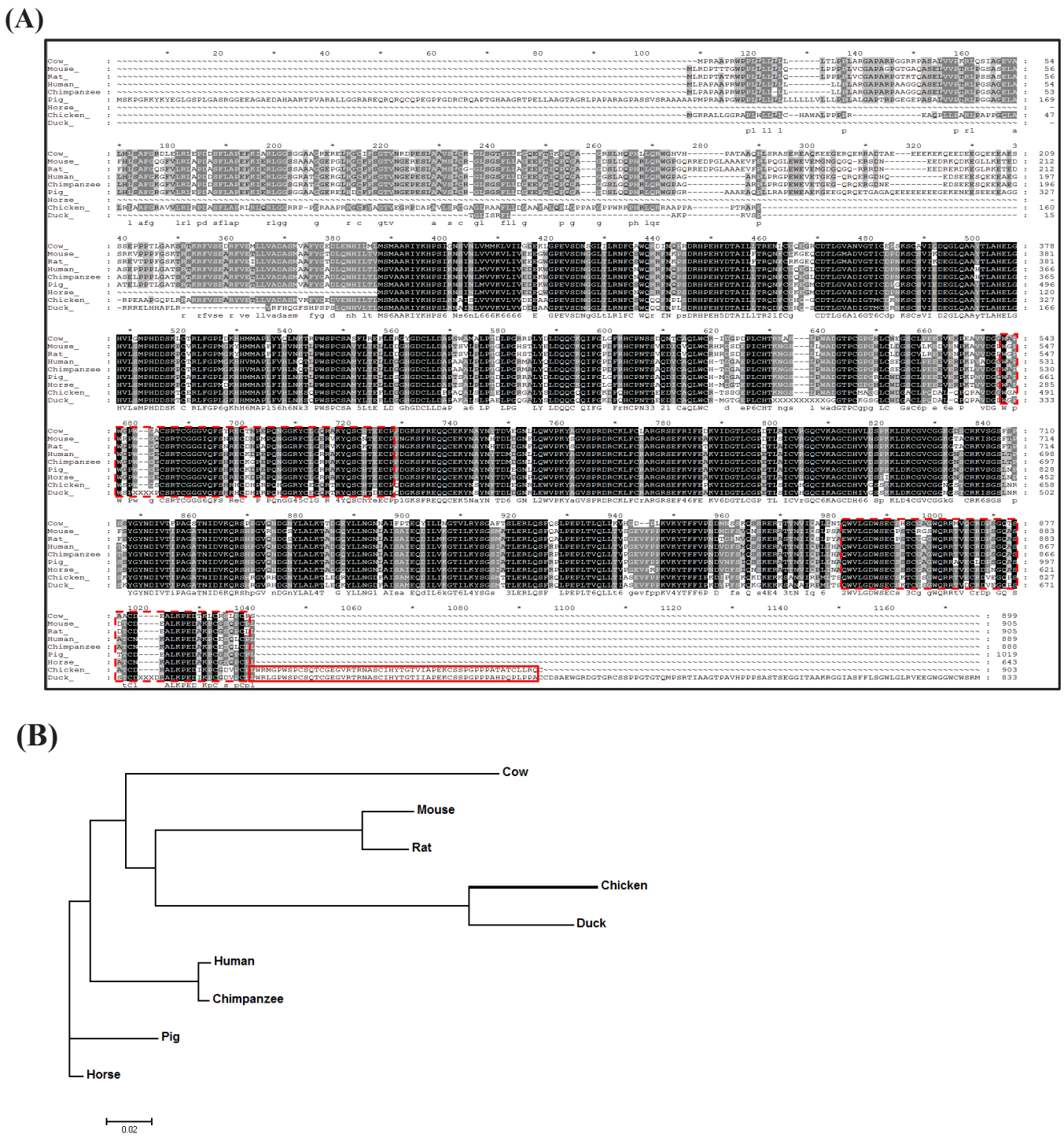

Figure 1. Analysis of a disintegrin and metallopeptidase with thrombospondin motifs type 8 (ADAMTS8) amino acid sequences. (A) Comparison of the chicken ADAMTS8 (chADAMTS8) amino acid sequence with those of various other species. ChADAMTS8 was predicted to comprise three thrombospondin type-1 (TSP1) domains. Two domains (dashed box) were conserved well among the species, while the additional third domain (solid box) was homologous only with the duck sequence. (B) Phylogenetic tree for ADAMTS8 from various species. Phylogenetic analyses were performed with the amino acid sequence of each species, using MEGA7 software. ChADAMTS8 clustered in the same clade with duck. The bar indicates 2% amino acid divergence.

(*chSOCS1*), a negative regulator of cytokine signaling in mammals, than chicken embryonic fibroblast cells, suggesting the dampened signaling activity of DF-1 cells in IFN-alpha (IFN-α) signaling pathways through SOCS1 by inhibiting Janus kinase (JAK)-signal transducers and activator of transcription (STAT) signaling axes [24]. Nonetheless, our results show that *chTLR3*, *chTLR4*, and *chIL1b* expressions, as TLR3 signal target genes, are induced in poly(I:C) stimulation, suggesting that TLR3

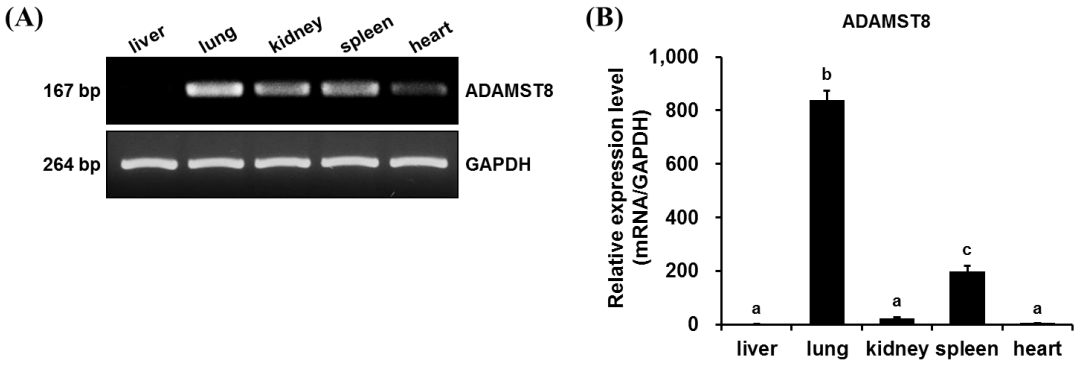

Figure 2. Expression of A disintegrin and metallopeptidase with thrombospondin motifs type 8 (*ADAMTS8*) in various chicken tissues. (A) Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *chADAMTS8* gene expression in various chicken tissues (liver, lung, kidney, spleen, and heart). (B) Quantitative PCR (qPCR) analysis of *chADAMTS8* expression in chicken tissues. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The values are presented as the mean±standard error of the mean. The expression levels of the gene varied among different tissues (n = 3, p<0.05). Significant differences were determined by Tukey's test, and bars with the same letter indicate cases where significant differences were not found (alpha = 0.05).

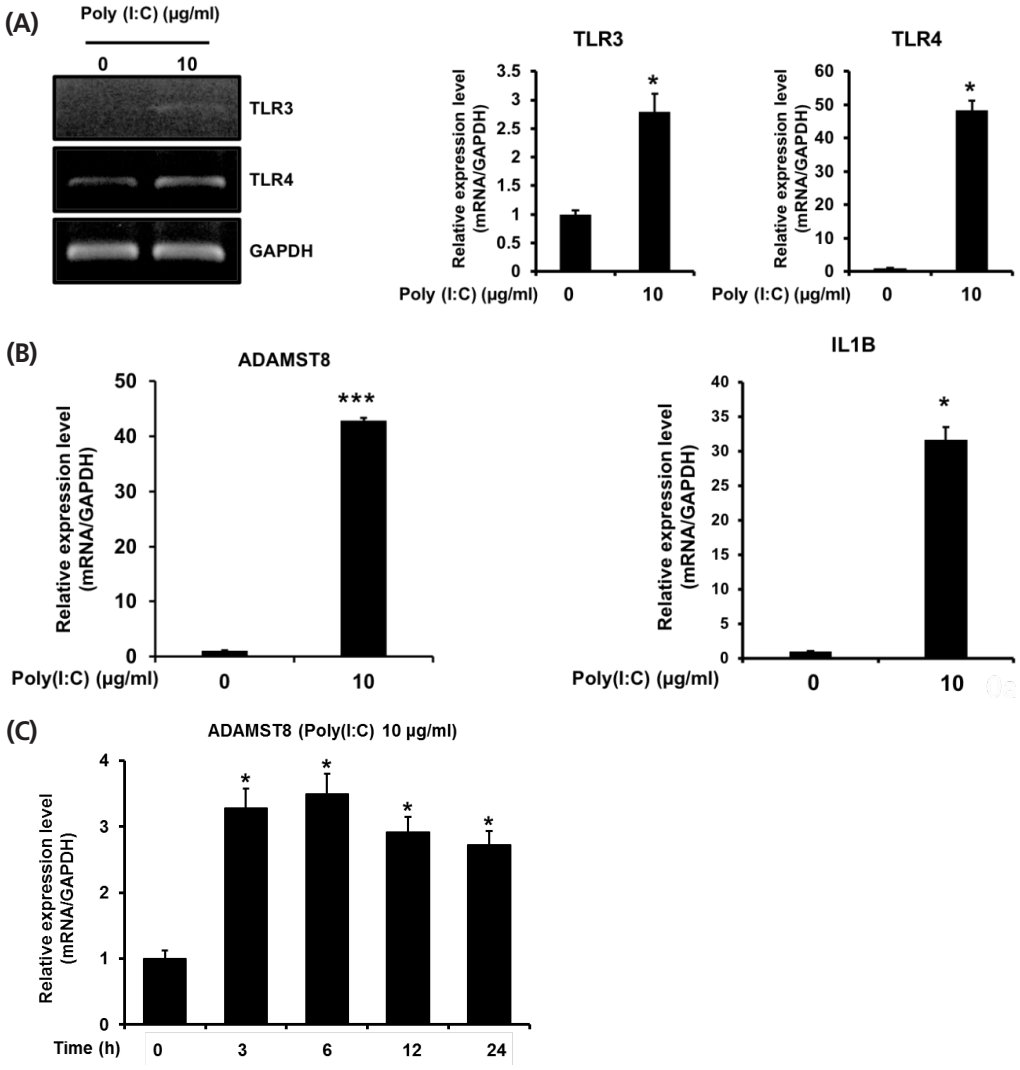

Figure 3. (A) Analyses of *chTLR3*, *chTLR4*, *chIL1B*, and *chADAMTS8* expression in polyinosinic-polycytidylic acid (poly[I:C]) treated DF-1 cells by quantitative polymerase chain reaction (qPCR). Expression of *chTLR3* and *chTLR4*, (B) *chADAMTS8* and *chIL1B* in chicken DF-1 cells after poly(I:C) treatment. (C) DF-1 cells were treated with 10 μg/mL of poly(I:C) for 24 h (n = 3, * p<0.05). Time dependent expression of *chADAMTS8* in poly(I:C) treated DF-1 cells. *chTLR*, chicken toll-like receptor; *chIL1B*, chicken interleukin 1 beta; *chADAMTS8,* chicken a disintegrin and metallopeptidase with thrombospondin motifs type 8.

signaling pathway of DF-1 cells may be separate from JAK-STAT signaling pathway activated by IFN-α,β, although further study is required to address it. Figure 3C shows qRT-PCR results of *ADAMTS8* expression in chicken DF-1 cells with the treatment of poly(I:C). After TLR3/Mda5 agonist poly(I:C) stimulation, *ADAMTS8* expression was measured time-dependently at 3, 6, 12, and 24 h. The expressional level of *chADAMTS8* was increased as early as 3 h, and after that, the level was not elevated further until 24 h. Overall, these results suggest that *chADAMTS8* expression may respond specifically to viral infection and thus, be correlated with TLR3-mediated cellular responses.

The complete molecular characterization of ADAMTS8 will be challenging; however, the genetic information and expression patterns of *chADAMTS8* presented here can provide the foundation for further study including the transcriptional inhibition effects on the *chADAMTS8* expression under the agonists poly(I:C). In addition, the up-regulation of *chADAMTS8* after poly(I:C) treatment suggests that this gene might be related to viral infection in chickens and that *chADAMTS8* gene is a promising candidate biomarker for infectious diseases that are rampant in chickens.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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