NOTE
Immunology

Molecular characterization and immune responsive expression of feline MDA5 gene

Ryota KOBA[1]*, Sakurako SHOJI[1], Sawami YOSHIMURA[1] and Yukinobu TOHYA[1]

[1]Laboratory of Veterinary Microbiology, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Kameino 1866, Fujisawa, Kanagawa 252-0880, Japan

ABSTRACT. The retinoic acid-inducible gene-I-like receptor (RLR) family is a group of cytosolic RNA helicase proteins that play an important role in sensing viral RNAs. Melanoma differentiation-associated gene 5 (MDA5), an RLR protein, recognizes viral double-stranded RNA and 5′-triphosphate single-stranded RNA in the cytoplasm for the expression of type I interferon (IFN). The expression of MDA5 is also induced by type I IFN. In the present study, we determined the complete coding sequence of the feline MDA5 gene, and analyzed its structure. In addition, we examined tissue expression patterns, inducibilities of the feline MDA5 by polyinosinic-polycytidylic acid and type I IFN, and a functional role of feline MDA5 on type I IFN expression.

KEY WORDS: cat, innate immunity, interferon, Melanoma differentiation-associated gene 5 (MDA5)

The innate immune system is the first line of defense against penetrating pathogens. Several ligands, including products released from pathogens, known as pathogen associated molecular patterns (PAMPs), have been recognized by pattern recognition receptors such as Toll-like receptors, NOD-like receptors, and cytoplasmic helicase proteins [1, 4, 5, 7, 16]. Melanoma differentiation-associated gene 5 (MDA5), known as a member of the retinoic acid-inducible gene-I-like receptor (RLR) family, recognizes nucleic acid PAMPs [8].

Previous studies have revealed that the recognition of nucleic acid PAMPs by MDA5 plays a critical role in the intracellular signal transduction pathway that could activate the interferon (IFN)-β promoter and mediate type I IFN responses against viral infection [6, 10]. MDA5 has five functional domains: two caspase recruitment domains (CARDs), a DExD-like helicase domain, a Helicase C domain, and a C-terminal regulatory domain (RD). The CARD region is used for signal transduction through CARD–CARD interaction between MDA5 and mitochondrial antiviral signaling protein (MAVS) [11, 23]. Upon viral invasion, MDA5 recruits the adaptor protein-MAVS. MAVS interacts with TNF receptor-associated factor 3 (TRAF3) and TANK-binding kinase-1 (TBK1) to activate interferon regulatory factor 3 (IRF3) by phosphorylation. The activated IRF3 enters the nucleus to induce the expression of type I IFN genes, resulting in an antiviral state in host cells [22]. It has been reported that long double-stranded RNA (dsRNA), including dsRNA replication intermediates of positive-sense RNA viruses and the genomes of dsRNA viruses, and polyinosinic-polycytidylic acid (poly I:C) are recognized by MDA5 [10, 18].

Zou *et al*. reported that MDA5 is conserved throughout vertebrate species including fish, birds and mammals [24]. Moreover, MDA5 is expressed at low levels in many cell types, and its expression is further induced by type I IFN, poly I:C, and viral challenge [9, 20, 22]. In contrast to the MDA5 gene of other vertebrates, feline MDA5 (feMDA5) has not been identified and characterized.

In the present study, the predicted amino acid sequence of feMDA5 was determined and characterized in terms of its protein structure and relationship with other mammalian MDA5 proteins and feMDA5 expression in several tissues. Furthermore, we analyzed feMDA5 inducibility by type I IFN and poly I:C in feline cell lines and a functional role of feline MDA5 on type I IFN expression.

Total RNA for sequence determination of feMDA5 was extracted from Crandell feline kidney (CRFK) cells using the RNeasy Plus Mini Kit (QIAGEN, Venlo, Netherlands). Complementary DNA (cDNA) was synthesized using the PrimeScript first-strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. Partial putative sequences of orthologous feMDA5 were identified in the feline genome using the GARField (http://lgd.abcc.ncifcrf.gov/) and UCSC (http://genome.ucsc.edu/) genome browser databases. Primers were designed on the basis of these data. The primers used to amplify the open reading frame of feline MDA5 gene were as follows: forward primer feMDA5-full-F, 5′-CCC TCC CTT TTC TGA GCA CGA ACG AA-3′ and reverse primer feMDA5-full-R, 5′-TCC TCA CAT CAG TTC TGT AGT ATA GTA A-3′. PCR was carried using TAKARA LA Taq polymerase (Takara Bio) and the reaction was performed as follows: initial incubation at 98°C for 2

min, followed by 40 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, extension at 72°C for 3 min, and a final extension at 72°C for 7 min. The PCR products were visualized by electrophoresis on 2% agarose gel stained with SYBR Safe (Life Technologies, Carlsbad, CA, U.S.A.). The amplified feMDA5 sequences was cloned by inserting the PCR product into the pCR2.1 TOPO vector (Life Technologies). The 5′ end was determined using the RACE method with the 5′-Full Race Core Set (Takara Bio) and pCR 2.1 TOPO vector according to the manufacturer's instructions. Oligo dT primer was used for the amplification of the 3′ end. The feMDA5 sequence was analyzed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). The feMDA5 putative sequence obtained from the feline cell line consisted of a 3081-bp open reading frame encoding a 1026-amino-acid protein (Genbank accession no. LC228059.2). A phylogenetic tree was generated to analyze the evolutionary relationship of feMDA5 protein with MDA5 protein of other mammalian species (Fig. 1). FeMDA5 was more closely related to dog and giant panda MDA5 proteins than to rodent MDA5 proteins. Similar to other mammals, the feMDA5 protein contained five main structural domains: two CARDs (amino acid

Fig. 1. Phylogenetic tree showing the evolutionary relationship of MDA5 by the neighbor-joining method. Phylogenetic analysis was performed using MEGA7 software. Numbers indicate bootstrap values of 1,000 replications. The zebrafish MDA5 sequence was used as an outgroup.

residues 7–96 and 116–203), DExD-like helicase (amino acid residues 308–522), helicase C-terminal domain (amino acid residues 706–823), and RD (amino acid residues 900–1016) (Fig. 2). CARD plays a critical role in signal transduction in the RLR pathway [12], and DExD/H helicase is known to be a domain for the recognition of viral RNA for ATPase activity [2]. The RD region acts as an RNA-binding site involved in auto-regulation [21]. The putative amino acid sequence of feMDA5 exhibited high similarities to that of other vertebrates, with conserved functional domains including two CARDs, a DExD-like helicase, helicase C, and RD (Fig. 2). Moreover, each of the four domains in MDA5 shows abundance of consensus amino acids, although feMDA5 is more genetically distant to human, cattle, and mouse MDA5 than to giant panda and dog MDA5. Therefore, it is proposed that these domains of feMDA5 have been highly conserved throughout evolution. Taken together, these results indicate that the structure and biological functions of feMDA5 are similar to those of MDA5 of other vertebrate species. However, as the functions of feMDA5 domains remain unclear, further studies are required to clarify the functional mechanisms of feMDA5 and its domains.

In order to detect feMDA5 transcripts in tissues, cDNA was synthesized from 100 ng of total RNA extracted from various tissues and organs (thymus, lymph node, liver, spleen, kidney, and heart) of two healthy male cats (2-month-old kitten and 5-year-old adult). PCR was carried using TAKARA Ex Taq polymerase (Takara Bio) and the reaction was performed as follows: initial incubation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 7 min. The primers used to amplify the MDA5 gene were as follows: forward primer feMDA5-F, 5′- CTG GTT TTC TGC ATT TCT GAC TG-3′ and reverse primer feMDA5-R, 5′- TCC GTG TCC CGA CCT TCT TT -3′. The feline β-actin primers were obtained from a previous study [13] and were as follows: forward primer feActin-F, 5′- AAG GCC AAC CGT GAG AAG AT -3′ and reverse primer feActin-R, 5′- TCC CCA GAG TCC ATG ACA AT -3′. The expression of the MDA5 gene was detected in all tissues obtained from the kitten and adult cat, although the expression levels varied among tissues. The MDA5 gene was expressed at lower levels in the spleen and kidney than in the other tissues (Fig. 3). Because the expression levels of feMDA5 were not uniform, the distribution pattern of feMDA5 was considered to be organ-specific. Previous studies have reported that MDA5 is involved not only in antiviral immunity, but also in tumor differentiation and apoptosis [8, 14]. Moreover, MDA5 is responsible for the pathogenesis of several immune disorders [4, 15, 17, 19]. Although the roles of the feMDA5 gene identified in each tissue were not clarified in the present study, it might have some pathophysiological effects on the organ in which it is expressed.

To investigate the inducibility of feMDA5 gene in feline cells stimulated with type I IFN or poly I: C, two feline cell lines were selected. CRFK cells and *Felis catus* whole fetus (Fcwf)-4 cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. For stimulation experiments, 2×10^5 cells were cultured in a 24-well plate with 1,000 unit/ml of feline IFN-ω (TORAY, Tokyo, Japan) as the type I IFN or 500 ng/ml of poly I:C (Tocris Bioscience, Bristol, U.K.). Template cDNA was synthesized from 500 ng of total RNA using the PrimeScript RT reagent Kit (Takara Bio) according to the manufacturer's instructions. Analysis of mRNA levels of the feMDA5 gene in cells treated with IFN-ω was performed by quantitative PCR (qPCR) using the Thermal Cycler Dice Real Time System Lite (Takara Bio). The initial qPCR reaction was performed at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 40 sec. After the stimulation of Fcwf-4 cells with poly I:C for 2–12 hr, the MDA5 transcripts showed an increase in the expression levels in comparison with the control values (Fig. 4). MDA5 expression stimulated by poly I:C was observed 2 hr post stimulation, and the peak level showed a 49-fold induction. Previous studies have shown that MDA5 expression levels are clearly upregulated after poly I: C stimulation in macrophages [3]. Our results were consistent with this finding, suggesting that feMDA5 expression is induced by dsRNA produced during viral replication. As shown in Fig. 5A and 5B, induction of the expression of MDA5 gene began 2 hr after IFN stimulation, and gradually increased until 4 hr in CRFK cells and 8 hr in Fcwf-4 cells. The peak levels showed a 21.0- and 31.4-fold induction
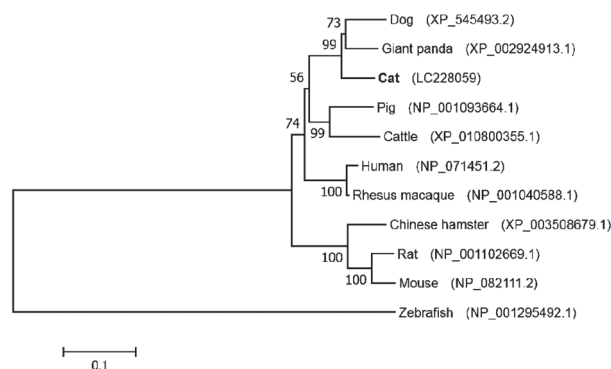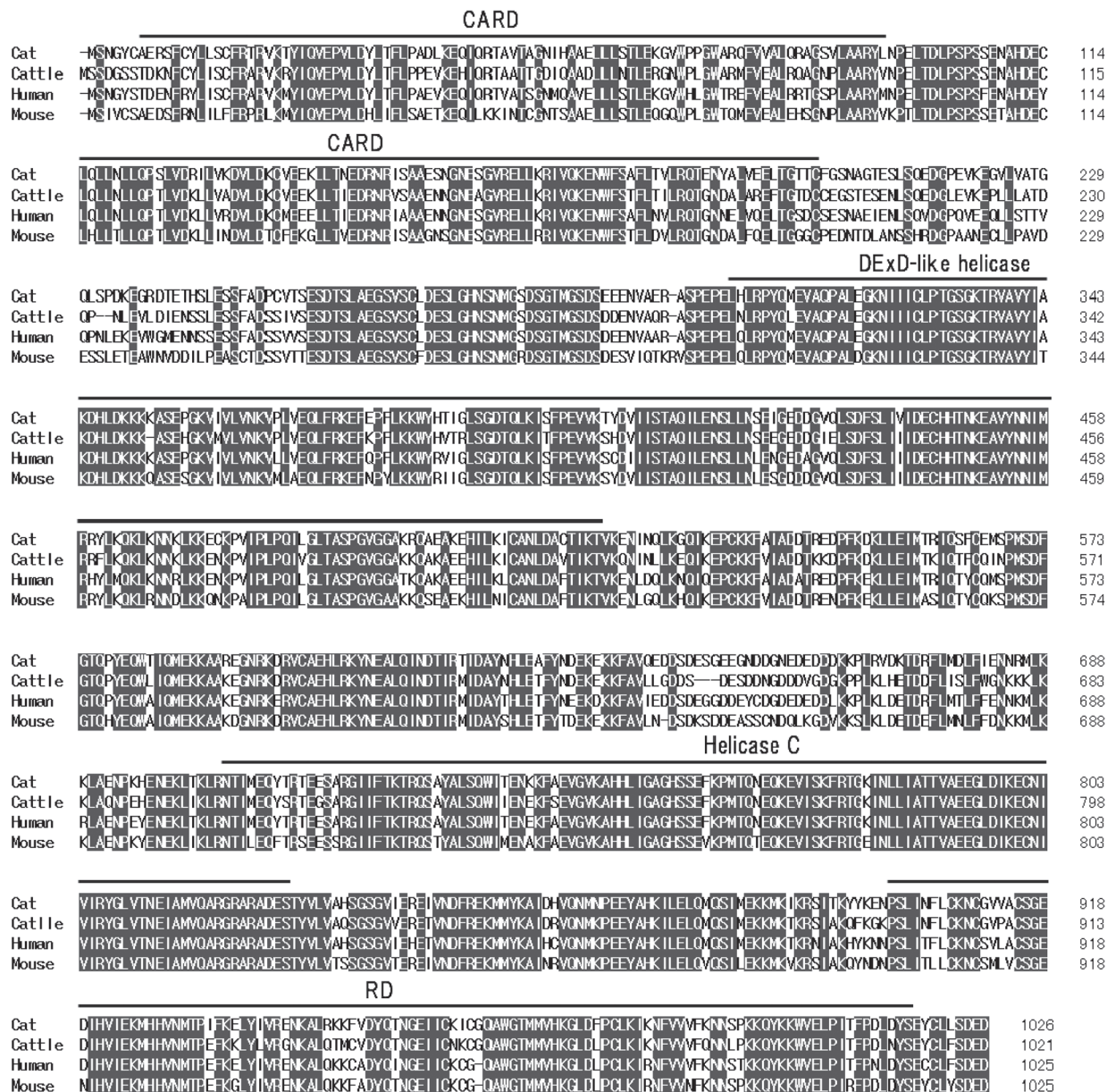
Fig. 2. Amino acid sequence alignment of the MDA5 proteins. Prediction of the conserved domains was performed using SMART program (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.xfam.org/). The predicted domains are indicated with lines on the alignment. Identical residues are in gray boxes.
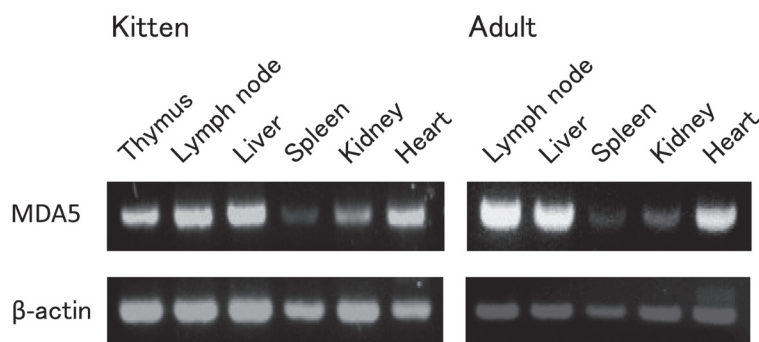

Fig. 3. Representative results of semi-quantitative RT-PCR of MDA5 gene expression in various feline tissues. β-actin was amplified as a housekeeping gene.
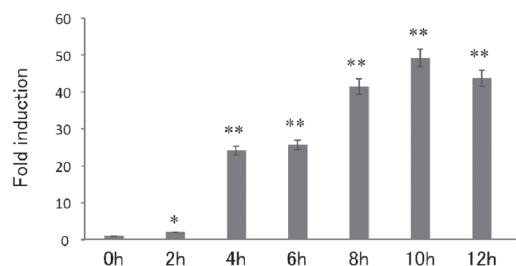
Fig. 4. The expression of the MDA5 gene in Fcwf-4 cells stimulated by poly I:C. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method using Thermal Cycler Dice Real Time System Lite Software Ver.5.11 (Takara Bio). The relative mRNA expression levels of feMDA5 in each sample were expressed as the mean ± standard deviation, and were statistically analyzed by Student's *t*-test. Data are presented as fold induction compared with untreated cells. Asterisks denote significant differences (*$P<0.05$, **$P<0.01$).
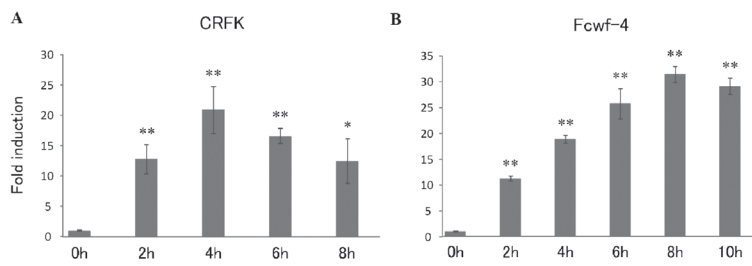

Fig. 5. The expression of the MDA5 gene in CRFK and Fcwf-4 cells stimulated by IFN-ω (A and B). All data were analyzed by the $2^{-\Delta\Delta Ct}$ method using Thermal Cycler Dice Real Time System Lite Software Ver.5.11 (Takara Bio). The relative mRNA expression levels of feMDA5 in each sample were expressed as the mean ± standard deviation, and were statistically analyzed by Student's *t*-test. Data are presented as fold induction compared with untreated cells. Asterisks denote significant differences (*$P<0.05$, **$P<0.01$).

in CRFK and Fcwf-4 cells, respectively. In a previous study, using northern blot analysis, it has been reported that human MDA5 induction by type I IFNs increases as time passes [9]. IFN-dependent expression of feMDA5 is consistent with that observed in previous studies, suggesting that feMDA5 is also responsible for the antiviral response mediated by IFN signaling in cats. However, because the induction levels and the time required to reach maximum induction, differed between the two cell lines, it can be considered that the IFN-mediated inducibility of the feMDA5 gene is depending on cell type-specific signaling.

To investigate whether exogenously feMDA5 stimulates the expression of IFN-β mRNAs in response to poly I:C, the plasmid expressing feMDA5 protein was prepared as follows. cDNA was synthesized from total RNA extracted from CRFK cells and was used to amplify the translated region of feMDA5 by RT-PCR using PrimeSTAR GXL polymerase (Takara Bio). The amplified feMDA5 sequence was cloned by the PCR products into the pCR2.0-TOPO vector (Life Technologies). Subsequently, the fragment containing feMDA5 was ligated into the site between *Xho*I and *Eco*RV of pcDNA3 vector (Life Technologies). Plasmid expressing human MDA5 (huMDA5) was kindly provided by Dr. Takemasa Sakaguchi at Hiroshima University. For stimulation experiments, Fcwf-4 cells prepared at 2×10^5 cells in a 24-well plate were transfected with 100 *n*g/well of plasmid-expressing MDA5 or pcDNA3 using FuGene HD (Promega, Madison, WI, U.S.A.). After 24 hr post-transfection, cells were further transfected with or without 500 *n*g/well of poly I:C. At 9 hr post-transfection, RNA extraction from the cells and cDNA synthesis was performed as described above. For analysis of mRNA levels of the IFN-β gene, qPCR was performed as described above. The primers used to amplify the IFN-β gene were as follows: forward primer feIFN-β-F, 5′- AAA AAT CAC AGC GGT TCC AG -3′ and reverse primer feIFN-β-R, 5′- CTC CTC CAT GAT TTC CTC CA -3′. FeMDA5 and huMDA5 increased IFN-β mRNA

Fig. 6. The effects of MDA5 on the expression of IFN-β in Fcwf-4 cells. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method using Thermal Cycler Dice Real Time System Lite Software Ver.5.11 (Takara Bio). The relative mRNA expression levels of IFN-β in each sample were expressed as the mean ± standard deviation, and were statistically analyzed by Student's *t*-test. Data are presented as fold induction compared with pcDNA3-transfected cells. Asterisks denote significant differences (*$P<0.01$).

production induced by poly I:C to 3.5- and 2.5- fold of the empty pcDNA3 vector, respectively (Fig. 6). The induction level of IFN-β expression using feMDA5 expression plasmid was stronger than that of huMDA5 expression plasmid. The differential levels of IFN-β induction between feMDA5 and huMDA5 indicate that MDA5 exhibit species specificity. The IFN-β expression levels were not significantly different in the poly I:C-untreated cells transfected with each plasmid. Previous studies have shown that mouse MDA5 signaling is triggered by poly I:C, resulting in IFN-β expression [6, 10]. Our results were consistent with these findings, suggesting that feMDA5 could play similar role as observed with mouse MDA5.

In conclusion, we found that the feMDA5 protein was highly conserved throughout evolution. Gene expression analysis revealed the distribution pattern of feMDA5, and the gene was found to be induced by poly I:C and type I IFN in feline cells. In addition, overexpression of feMDA5 enhanced the poly I:C-dependent expression of IFN-β. Overall, our results indicate that feMDA5 can
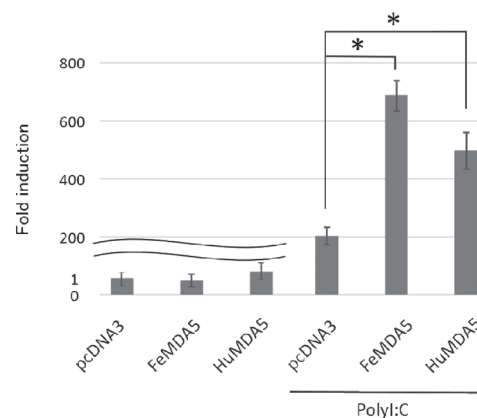
sense dsRNA, resulting in IFN-β expression, suggesting its functional importance as an RLR in feline immunity. Further studies are required to elucidate the role of MDA5 in antiviral immune responses and the signaling pathways involved in the induction of the expression of the MDA5 gene by type I IFN and viral RNA.

REFERENCES

1. Akira, S., Uematsu, S. and Takeuchi, O. 2006. Pathogen recognition and innate immunity. *Cell* **124**: 783–801. [Medline] [CrossRef]
2. Bamming, D. and Horvath, C. M. 2009. Regulation of signal transduction by enzymatically inactive antiviral RNA helicase proteins MDA5, RIG-I, and LGP2. *J. Biol. Chem.* **284**: 9700–9712. [Medline] [CrossRef]
3. Chang, M., Collet, B., Nie, P., Lester, K., Campbell, S., Secombes, C. J. and Zou, J. 2011. Expression and functional characterization of the RIG-I-like receptors MDA5 and LGP2 in Rainbow trout (Oncorhynchus mykiss). *J. Virol.* **85**: 8403–8412. [Medline] [CrossRef]
4. Chen, G., Shaw, M. H., Kim, Y. G. and Nuñez, G. 2009. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu. Rev. Pathol.* **4**: 365–398. [Medline] [CrossRef]
5. Franchi, L., Muñoz-Planillo, R., Reimer, T., Eigenbrod, T. and Núñez, G. 2010. Inflammasomes as microbial sensors. *Eur. J. Immunol.* **40**: 611–615. [Medline] [CrossRef]
6. Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R. A., Diamond, M. S. and Colonna, M. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc. Natl. Acad. Sci. U.S.A.* **103**: 8459–8464. [Medline] [CrossRef]
7. Hansen, J. D., Vojtech, L. N. and Laing, K. J. 2011. Sensing disease and danger: a survey of vertebrate PRRs and their origins. *Dev. Comp. Immunol.* **35**: 886–897. [Medline] [CrossRef]
8. Kang, D. C., Gopalkrishnan, R. V., Wu, Q., Jankowsky, E., Pyle, A. M. and Fisher, P. B. 2002. *mda-5*: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc. Natl. Acad. Sci. U.S.A.* **99**: 637–642. [Medline] [CrossRef]
9. Kang, D. C., Gopalkrishnan, R. V., Lin, L., Randolph, A., Valerie, K., Pestka, S. and Fisher, P. B. 2004. Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, *mda-5*: a novel type I interferon-responsive apoptosis-inducing gene. *Oncogene* **23**: 1789–1800. [Medline] [CrossRef]
10. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S., Reis e Sousa, C., Matsuura, Y., Fujita, T. and Akira, S. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**: 101–105. [Medline] [CrossRef]
11. Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O. and Akira, S. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* **6**: 981–988. [Medline] [CrossRef]
12. Kawai, T. and Akira, S. 2008. Toll-like receptor and RIG-I-like receptor signaling. *Ann. N. Y. Acad. Sci.* **1143**: 1–20. [Medline] [CrossRef]
13. Koba, R., Kokaji, C., Fujisaki, G., Oguma, K. and Sentsui, H. 2013. Characterization of feline TRIM genes: molecular cloning, expression in tissues, and response to type I interferon. *Vet. Immunol. Immunopathol.* **153**: 91–98. [Medline] [CrossRef]
14. Kovacsovics, M., Martinon, F., Micheau, O., Bodmer, J. L., Hofmann, K. and Tschopp, J. 2002. Overexpression of Helicard, a CARD-containing helicase cleaved during apoptosis, accelerates DNA degradation. *Curr. Biol.* **12**: 838–843. [Medline] [CrossRef]
15. Martínez, A., Santiago, J. L., Cénit, M. C., de Las Heras, V., de la Calle, H., Fernández-Arquero, M., Arroyo, R., de la Concha, E. G. and Urcelay, E. 2008. *IFIH1-GCA-KCNH7* locus: influence on multiple sclerosis risk. *Eur. J. Hum. Genet.* **16**: 861–864. [Medline] [CrossRef]
16. Meylan, E., Tschopp, J. and Karin, M. 2006. Intracellular pattern recognition receptors in the host response. *Nature* **442**: 39–44. [Medline] [CrossRef]
17. Nejentsev, S., Walker, N., Riches, D., Egholm, M. and Todd, J. A. 2009. Rare variants of *IFIH1*, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science* **324**: 387–389. [Medline] [CrossRef]
18. Reikine, S., Nguyen, J. B. and Modis, Y. 2014. Pattern recognition and signaling mechanisms of RIG-I and MDA5. *Front. Immunol.* **5**: 342. [Medline] [CrossRef]
19. Robinson, T., Kariuki, S. N., Franek, B. S., Kumabe, M., Kumar, A. A., Badaracco, M., Mikolaitis, R. A., Guerrero, G., Utset, T. O., Drevlow, B. E., Zaacks, L. S., Grober, J. S., Cohen, L. M., Kirou, K. A., Crow, M. K., Jolly, M. and Niewold, T. B. 2011. Autoimmune disease risk variant of IFIH1 is associated with increased sensitivity to IFN-α and serologic autoimmunity in lupus patients. *J. Immunol.* **187**: 1298–1303. [Medline] [CrossRef]
20. Su, J., Huang, T., Dong, J., Heng, J., Zhang, R. and Peng, L. 2010. Molecular cloning and immune responsive expression of *MDA5* gene, a pivotal member of the RLR gene family from grass carp *Ctenopharyngodon idella*. *Fish Shellfish Immunol.* **28**: 712–718. [Medline] [CrossRef]
21. Takahasi, K., Yoneyama, M., Nishihori, T., Hirai, R., Kumeta, H., Narita, R., Gale, M. Jr., Inagaki, F. and Fujita, T. 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Mol. Cell* **29**: 428–440. [Medline] [CrossRef]
22. Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S. and Fujita, T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5**: 730–737. [Medline] [CrossRef]
23. Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M. Jr., Akira, S., Yonehara, S., Kato, A. and Fujita, T. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **175**: 2851–2858. [Medline] [CrossRef]
24. Zou, J., Chang, M., Nie, P. and Secombes, C. J. 2009. Origin and evolution of the RIG-I like RNA helicase gene family. *BMC Evol. Biol.* **9**: 85. [Medline] [CrossRef]