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RESEARCH ARTICLE

First Report of *IFITM3* Polymorphism Associated with Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV) Infection in the Raccoon Dog

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ABSTRACT

Severe fever with thrombocytopenia syndrome virus (SFTSV), a novel *Phlebovirus* within the family *Phenuiviridae*, is the causative agent of severe fever with thrombocytopenia syndrome (SFTS), a tick-borne zoonotic disease. Raccoon dogs (*Nyctereutes procyonoides*) have been identified as potential reservoirs of SFTSV. Interferon-induced transmembrane protein 3 (*IFITM3*) plays a critical role in the host antiviral response and has been implicated in restricting SFTSV infection. This study investigated the association between *IFITM3* gene polymorphisms and susceptibility to SFTSV infection in raccoon dogs. Genotype, allele, and haplotype frequencies were compared between healthy and SFTSV-infected animals. In addition, *in silico* programs were used to evaluate the functional impact of a 3′ untranslated region (UTR) single-nucleotide polymorphism (SNP) (c.447+34G>A) and a non-synonymous SNP (c.52C>T, P18S). Furthermore, the 3D structure modeling was performed to assess structural alterations associated with the P18S variant. A significant difference in genotype frequency of the c.447+34G>A SNP was observed between healthy and SFTSV-infected raccoon dogs. RNAfold and CentroidFold predict that this SNP (c.447+34G>A) affects RNA structure and energy. Functional predictions from PolyPhen-2 and SIFT indicated that the c.52C>T (P18S) substitution may be deleterious, although E-SNPs & GO classified it as benign. Structural modeling suggested that the P18S variant alters local hydrogen bonding, potentially affecting protein stability and flexibility. This study presents the first genetic association analysis of *IFITM3* polymorphisms in raccoon dogs. Our findings suggest a potential link to SFTSV susceptibility, emphasizing the potential role of host genetic variation in modulating SFTSV susceptibility.

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INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne disease caused by the SFTS virus (SFTSV), a negative-sense RNA virus. First reported in Asia, it is characterized by high fever, vomiting, and thrombocytopenia (Yu *et al*., 2011; Kim *et al*., 2013; Liu *et al*., 2014; Li, 2015). Although SFTSV is transmitted to humans by tick bites (Yu *et al.*, 2011), it can be sporadically transmitted through oral and ocular membranes (Zhou *et al.*, 2022) and via direct contact with the body fluids of SFTS patients (Li *et al.*, 2024). There have been reports of direct transmission of the SFTSV from animals (dogs and cats) to humans (Chung *et al.*, 2020; Yamanaka *et al.*, 2020), emphasizing that SFTS is a zoonotic disease that can be transmitted without the involvement of tick bites, which presents an additional public health issue. Instances of suspected SFTSV transmission from domestic dogs to humans have been documented in the Republic of Korea (Chung *et al.*, 2020; Kim *et al.*, 2025).

Interferon-induced transmembrane protein 3 (*IFITM3*) protein, a member of the family of interferon-induced transmembrane proteins (*IFITMs*), plays a

significant role in the host innate immune response against viruses (Friedlová *et al.*, 2022). A previous study indicated that both interferon-induced transmembrane protein 1 (*IFITM1*) protein and *IFITM3* protein of the monkey could effectively restrict SFTSV infection, with *IFITM3* protein demonstrating a more pronounced viral inhibition rate compared to *IFITM1* protein (Xing *et al.*, 2020). These results indicate that *IFITM* family members exhibit distinct functions, which may depend on their structural characteristics and the types of pathogens. Another study reported that human *IFITM3* protein plays a vital role in the interferon-mediated response against SFTSV (Du *et al.*, 2024). Specifically, *IFITM3* exhibits strong resistance to various RNA viruses by inhibiting viral membrane fusion and intracellular propagation (Verma *et al.*, 2023; Aftab *et al.*, 2024). These findings highlight that *IFITM3* acts as a virus-entry inhibitor, playing a major role in the type I and III IFN-mediated anti-SFTSV response.

The complete genome sequences of two SFTSV strains isolated from the serum of a dog with asymptomatic SFTS infection have been investigated (Lee *et al.*, 2019). The potential for direct zoonotic transmission underscores the importance of understanding SFTSV in animal reservoirs. Raccoon dogs serve as the primary hosts for various infectious diseases, including canine distemper, rabies, and parasites, which can be transmitted between pet and wild Canidae species (Botvinkin *et al.*, 2006; Kauhala and Kowalczyk, 2011). This study focused on the raccoon dog because of its wide distribution across South Korea, primarily inhabiting forested and agricultural areas (Hong *et al*., 2018; Kim-Jeon *et al*., 2022).

Multiple surveillance studies have confirmed raccoon dogs as competent hosts and potential amplifying reservoirs for SFTSV in Korea. For instance, Oh *et al*. (2016) detected SFTSV RNA in 3.3% (3/91) of wild-caught mammals, including raccoon dogs, from SFTS-endemic areas. While SFTS has been reported in various species in Korea (Chen *et al*., 2019; Nam *et al*., 2020; Ji *et al*., 2024), genetic studies on raccoon dogs are lacking.

Host genetic variations may contribute to the severity and death of SFTS (Wang *et al.*, 2022). Since variations in the *IFITM3* gene play a significant role in immune responses, further fine mapping and identification of disease-related SNPs in the raccoon dog *IFITM3* gene are required.

Therefore, this study aimed to carry out the first comprehensive analysis of the *IFITM3* gene in raccoon dogs to identify relevant *IFITM3* SNPs associated with SFTSV infection. To achieve this, we amplified the *IFITM3* gene sequence using polymerase chain reaction (PCR) and performed a multiple sequence alignment of raccoon dog *IFITM3* protein sequences with those from various species. In addition, we performed amplicon sequencing of *IFITM3* to identify genetic polymorphisms. We investigated the genotype, allele, and haplotype frequencies and linkage disequilibrium (LD) of SNPs in the raccoon dog *IFITM3* gene. Furthermore, we evaluated structural changes in *IFITM3*-caused by a non-synonymous SNP in the raccoon dogs using *in silico* tools.

MATERIALS AND METHODS

Ethical statements and subjects: We obtained tissue samples from 89 raccoon dogs, including 24 infected with SFTSV, from the National Institute of Environmental Research in the Republic of Korea and the College of Veterinary Medicine at Jeonbuk National University (Chae *et al.*, 2023). The Institutional Animal Care and Use Committee (IACUC) of Jeonbuk National University approved all experimental protocols (CBNU 2020-083).

Genetic analysis of the raccoon dog *IFITM3* gene: We amplified the *IFITM3* gene using PCR with forward and reverse primers as follows: *IFITM3*–1F (GTCAAGGGTGCGGGGATTG) / *IFITM3*–1R (AGCCTCCCTGCAGGTACAG) and *IFITM3*–2F (GCCCCTGAGTCCCTGGTC) / *IFITM3*–2R (CTTCCCCACACGAGCCATC). We prepared PCR mixtures and ran them under conditions according to the manufacturer's manual (ABI, Foster City, CA, USA) as previously performed (Jo *et al*., 2022; Choi *et al*., 2024). The PCR mixture contained 1µL of each 10mM dNTP mix, 2.5µL of 10× *Taq* reaction buffer, 1 µL of each primer (10µM), and 0.2µL of *Taq* DNA polymerase (BIOFACT, Daejeon, Republic of Korea). The PCR was carried out under the following conditions: 95°C for 3 min for the denaturation step, 35 cycles of 95°C for 30 s, 63°C for 45 s, and 72°C for 1 min for annealing and extension steps, and 1 cycle of 72°C for 10 min for the final extension step. We purified the PCR products using the FavorPrep GEL/PCR Purification Kit (Favogen Biotech, Ping Tung, Taiwan). Sequencing results were visualized using FinchTV v1.4.0 (Geospiza Inc., Seattle, WA, USA). All sequence metadata generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) GenBank repository under accession number PX520827.

Multiple sequence alignments: We collected the *IFITM3* protein sequences from GenBank at the NCBI. The sequences were compared by ClustalW (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) (accessed on 23 July 2024) based on progressive alignment methods. It works by building a guide tree from initial pairwise comparisons and then progressively aligning the sequences based on that tree.

RNA analysis: To predict whether the SNP (c.447+34G>A) located in the 3′ untranslated region (3′ UTR) is related to microRNA, we utilized a microRNA database (accessed on 21 March 2025). miRBase (https://www.mirbase.org/) is the main public repository and online resource for microRNA sequences and annotation (Griffiths-Jones *et al.*, 2008).

To predict RNA secondary structures, we utilized RNAfold and CentroidFold. RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) predicts the minimum free energy (MFE) structure for an RNA sequence, enabling evaluation of mRNA secondary structures and ensemble conformations (Gruber *et al.*, 2008; Uddin *et al.*, 2015). CentroidFold (http://rtools.cbrc.jp/centroidfold/) predicts RNA secondary structure using the γ-centroid estimator (Sato *et al.*, 2009; Sahu *et al.*, 2025).

Prediction of *IFITM3* protein functional alterations: To analyze the effects of non-synonymous SNPs on raccoon

dog *IFITM3* protein, we used several *in silico* programs, including PolyPhen-2 v2.2 (http://genetics.bwh.harvard.edu/pph2/), SIFT v6.2.1 (https://sift.bii.a-star.edu.sg/), E-SNPs&GO (https://esnpsandgo.biocomp.unibo.it/), AMYCO (http://bioinf.uab.es/amycov04/), and SODA (http://old.protein.bio.unipd.it/soda/). PolyPhen-2 predicts the impact of amino acid changes based on the Position-Specific Independent Counts (PSIC) score. This tool represents three types: "probably damaging," "possibly damaging," and "benign" (Adzhubei *et al.*, 2013). SIFT can also predict whether an amino acid substitution will change protein function. With SIFT, a variant is considered "deleterious" if its score is under 0.05 (Ng and Henikoff, 2003). E-SNPs&GO can indicate whether the variation is associated with diseases (Manfredi *et al.*, 2022). AMYCO can predict the potential for aggregation into amyloids. Scores below 0.45 indicate a weak aggregation propensity (Iglesias *et al.*, 2019). SODA can predict the effects of amino acid variations on protein solubility (Paladin *et al.*, 2017).

3D structure analysis: The 3D structure of raccoon dog *IFITM3* was predicted using AlphaFold2 (https://colab.research.google.com/github/sokrypton/ColabFold/blob/v1.2.0/AlphaFold2.ipynb) (accessed on 24 October 2024). AlphaFold2 is an advanced protein structure prediction program developed by DeepMind, known for its high accuracy (Jumper *et al.*, 2021).

Statistical Analyses: We analyzed LD and haplotype with Haploview v 4.2 (Broad Institute, Cambridge, MA, USA) (Barrett *et al*., 2005), and assessed Hardy-Weinberg Equilibrium (HWE) for each SNP using a chi-square test. No missing genotype data were observed, and all 89 samples provided complete SNP calls across all loci. With 24 SFTSV-positive and 65 healthy raccoon dogs, the study provides approximately 70–80% power to detect moderate genetic effects (odds ratio≥3.0) for variants with a minor allele frequency (MAF)≥0.15 at α=0.05. Allele and genotype frequencies were compared between SFTSV-positive and SFTSV-negative raccoon dogs using Fisher's exact test. Ninety-five percent confidence intervals (95% CI) were calculated for all estimated odds ratios. Multiple testing was controlled using the Bonferroni correction.

RESULTS

Identification of the *IFITM3* gene sequence in raccoon dogs: Primers targeting the dog *IFITM3* gene sequence were designed according to the previous study (Lu *et al.*, 2021). The amino acid length of human *IFITM1* (Gene ID: 8519) is 125 residues, whereas that of dog (Gene ID: 483397) and raccoon dog is 126 residues (Gene ID: 129494312). For *IFITM3*, the amino acid lengths in humans (Gene ID: 10410) and raccoon dogs are 133 and 148, respectively (Fig. 1). Consistent with a previous study, the amino acid length of dog *IFITM3* was found to be 147 residues (Lu *et al.*, 2021). Alignment of the identified raccoon dog *IFITM3* gene sequence with the dog *IFITM3* gene sequence revealed a sequence homology of 92.62%.

Identification of novel polymorphisms in the raccoon dog *IFITM3* gene: We then aimed to identify genetic variation within our cohort of raccoon dogs. A total of 17 novel polymorphisms were identified in the raccoon dog *IFITM3* gene (Fig. 2). These include 15 SNPs: c.-251G>A, c.-179G>T, c.-37T>C, c.-32G>T, c.-27C>A in upstream region; c.52C>T (P18S), c.147C>T in exon1; c.399C>T in exon2; c.447+29C>T, c.447+34G>A, c.447+124G>A, c.447+146T>C, c.447+151G>C, c.447+180G>A, c.447+216C>T in downstream region. In addition, two insertion/deletion polymorphisms were identified: c.-67delA and c.430_435delCATGAC (Fig. 2C and D).

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Human (NP_003632.4)              IFITM1  ----------MHKEEHEVAVL-GPPPSTILPRSTVINIHSETSVPDHVV  38
Dog (XP_038279423.1)             IFITM1  ----------MDQDQYKVPETGAPQSMVPVTTTVINIRSDTVVPDHIV  38
Raccoon dog (XP_055157128.1)     IFITM1  ----------MDQDQYQVPETGAPQSMVPMTTTVINIRGDTVVPDHIV  38
Human (NP_066362.2)              IFITM3  MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVL-GAPHNPAPPTSTVIHIRSETSVPDHVV  59
Dog (Lu, Gang, et al.,2021)      IITM3   MSRAPRPLLPGARAAGPPTYEMLKEEHEVVVLGGAPQSAAPATTTVINIRGDTVVPDHVV  60
Raccoon dog (This study)         IFITM3  MSRSPRPLLPGACAAGPPTYEMLKEEQEVVVV-RAPQSTAPATTTVINIHGDTVVPDHVV  59

Human (NP_003632.4)              IFITM1  WSLFNTLFLNWCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTI  98
Dog (XP_038279423.1)             IFITM1  WSLFNTVFMNWCCLGIVAFAYSVKARDRKMVGDLTGAQSFASTARCLNISALVVGIILGI  98
Raccoon dog (XP_055157128.1)     IFITM1  WSLFNTVFMNWCCLGFVAFAYSVKARDRKMVGDLTGAQSFASTARCFNISALVVGIILGI  98
Human (NP_066362.2)              IFITM3  WSLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMTI  119
Dog (Lu, Gang, et al.,2021)      IFITM3  WSLFNTVFMNWCCLGFVAFAYSEKTRDRKMAGDLTGAQSFASTARCLNIWALVLGLLLTV  120
Raccoon dog (This study)         IFITM3  WSLFNTVFMNWCCLGFLAFAYSVKARDRKMVGDLTGAQSFASTARCLNIWALVLGLLLTV  119

Human (NP_003632.4)              IFITM1  GFILLLVFGSVTVYHIMLQIIQEKRGY-----  125
Dog (XP_038279423.1)             IFITM1  ISIVLLRIAFGAAYWALLQVMQERSRYH----  126
Raccoon dog (XP_055157128.1)     IFITM1  ISIVLLGMAYTMVSGALLQAMQERRRYH----  126
Human (NP_066362.2)              IFITM3  LLIVIPVLIF-QAYG-----------------  133
Dog (Lu, Gang, et al.,2021)      IFITM3  TFVILVSTGSLVIFETVSEM---VKHYGGS--  147
Raccoon dog (This study)         IFITM3  TFVILVSTGSLMIFETVSEM---VKHDHDGGS  148
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Fig. 1: Comparison of the amino acid sequences of *IFITM1* and *IFITM3* among human, dog, and raccoon dog.

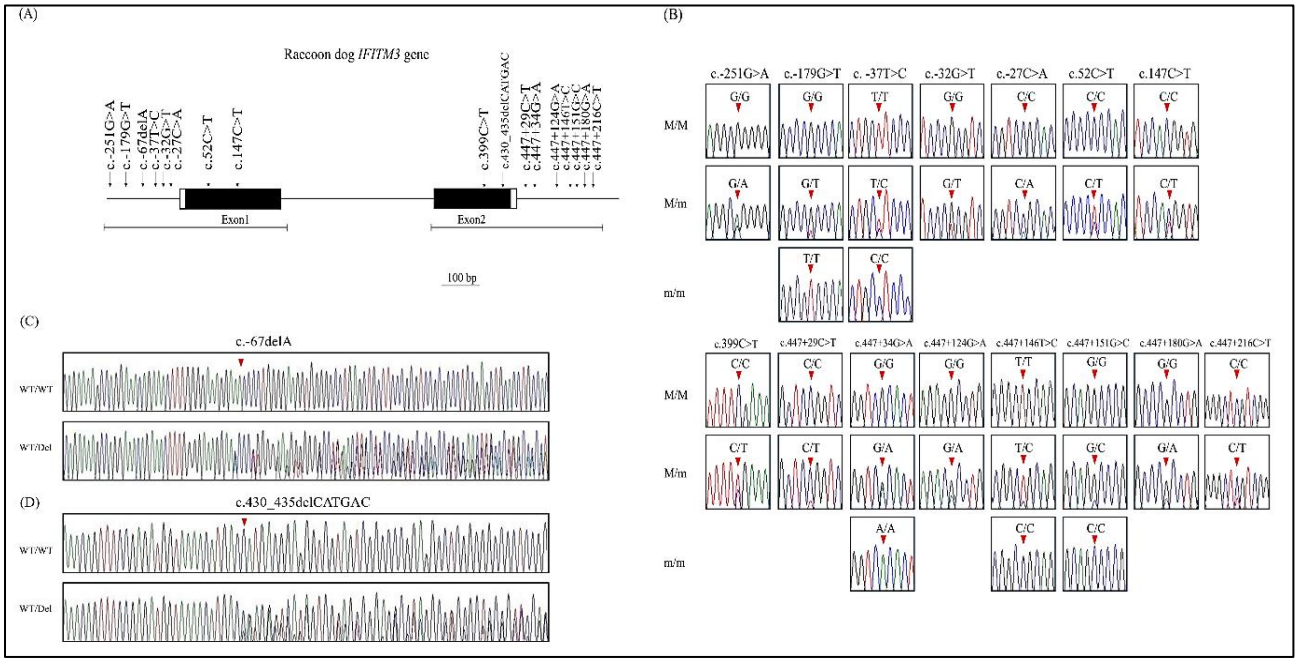

Fig. 2: Electropherograms of novel single-nucleotide polymorphisms (SNPs) found in the raccoon dog *IFITM3* gene. (A) Gene map and electropherograms of identified polymorphisms. The shaded block represents the open reading frame (ORF) within two exons. (B) Electropherograms of novel synonymous SNPs identified in exon 1 and exon 2 of the *IFITM3* gene. Red arrowheads indicate the precise nucleotide positions of the variants. M/M represents major homozygotes, M/m represents heterozygotes, and m/m represents minor homozygotes. (C) Electropherogram of c.-67delA polymorphism, with the deletion site marked by a red arrowhead. (D) Electropherogram of c.430_435delCATGAC polymorphism, with the deletion site marked by a red arrowhead.

Evaluation of the association between *IFITM3* polymorphisms and susceptibility to SFTSV infection in the raccoon dogs: A total of 89 raccoon dogs were included in the association analysis. Detailed information on the study is described in Table 1. To investigate whether polymorphisms in the *IFITM3* gene are associated with susceptibility to SFTSV infection, we compared the genotype and allele frequencies of the *IFITM3* polymorphisms between healthy controls and SFTSV-infected raccoon dogs. Among the 17 SNPs identified, c.447+34G>A exhibited a statistically significant difference in genotype frequencies between the two groups (P<0.05, Table 2 and Supplementary Table 1). Still, this association was not statistically significant after Bonferroni correction (adjusted P=0.336).

LD analysis of all SNPs in the raccoon dog *IFITM3* gene was conducted to determine their relationships, revealing twelve strong linkages (r^2>0.333) (Table 3). Haplotype frequencies were analyzed and are summarized in Table 4. Four major haplotypes with frequencies higher than 5% were identified. The GGITGCTCCDCGATGGC haplotype was the most prevalent. However, statistical comparisons of these four major haplotypes showed no significant differences in distribution between healthy controls and SFTSV-infected raccoon dogs (P>0.2).

Comparative analysis of the *IFITM3* protein sequence among species: To interpret our genetic findings, we conducted a comparative analysis to determine if key functional domains of the *IFITM3* protein are conserved in the raccoon dog. We performed multiple sequence alignments of the *IFITM3* protein from various species (Fig. 3). The YXXΦ motif, which facilitates *IFITM3* internalization, was conserved in all species except the mole. Two cysteine residues highlighted in red boxes known to undergo palmitoylation were conserved across species. In addition, three lysine residues shown in green boxes, corresponding to known ubiquitination sites, were consistently present in the aligned sequences (Fig. 3).

Table 1: The detailed information of this study population.

	Controls	Cases
Number	65	24
Sex (n, %)		
Male	36 (55.38)	8 (33.33)
Female	29 (44.62)	16 (66.67)

Values are presented as number (n) and percentage (%).

Table 2: Comparison of genotype and allele frequencies of the *IFITM3* polymorphisms between healthy controls and SFTS virus (SFTSV)-infected raccoon dogs.

Variant	Genotype P-value	Allele P-value
c.-251G>A	0.778	0.791
c.-179G>T	0.858	1
c.-67delA	1	1
c.-37T>C	0.409	0.379
c.-32G>T	1	1
c.-27C>A	1	1
c.52C>T (P18S)	1	1
c.147C>T	0.751	0.563
c.399C>T	0.347	0.329
c.430_435delCATGAC	0.469	1
c.447+29C>T	1	1
c.447+34G>A	P<0.05	0.577
c.447+124G>A	1	1
c.447+146T>C	0.267	0.672
c.447+151G>C	0.473	1
c.447+180G>A	1	1
c.447+216C>T	0.751	0.762

Characterization of SNP (c.447+34G>A): Since the c.447+34G>A SNP in the 3′ UTR was the only variant linked to infection, we investigated its possible functional effects. We examined the sequence surrounding the SNP (c.447+34G>A) located in the 3′ UTR region using

miRBase. As a result, it was similar to the dog microRNA cfa-mir-207 precursor (Gene ID: 100886181), showing an identity of 78.57% (Fig. 4A). Based on the analysis of secondary structures, the SNP (c.447+34G>A) was predicted to induce consistent RNA structural changes across *in silico* tools (Fig. 4B and C). RNAfold predicted a MFE of -105.80 kcal/mol for the G allele and -105.50 kcal/mol for the A allele.

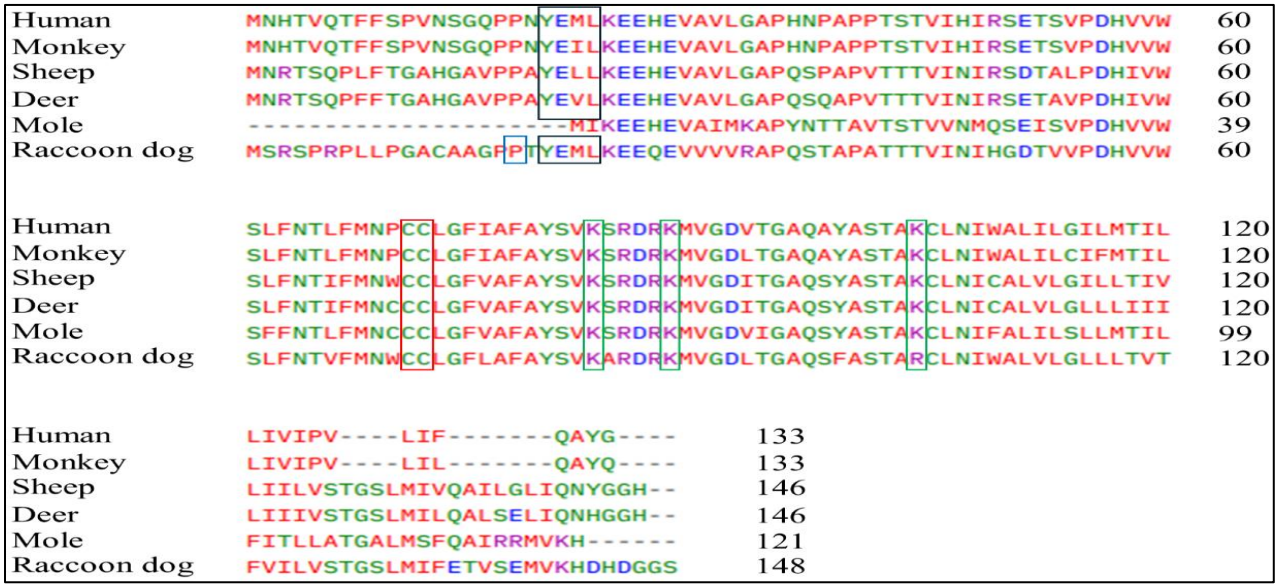

Fig. 3: Multiple sequence alignment of *IFITM3* amino acid sequences in various species. The blue box indicates a non-synonymous SNP. The black box indicates the YXXΦ motif. The two palmitoylated cysteines and three lysines serving as ubiquitination sites are indicated with red and green, respectively.

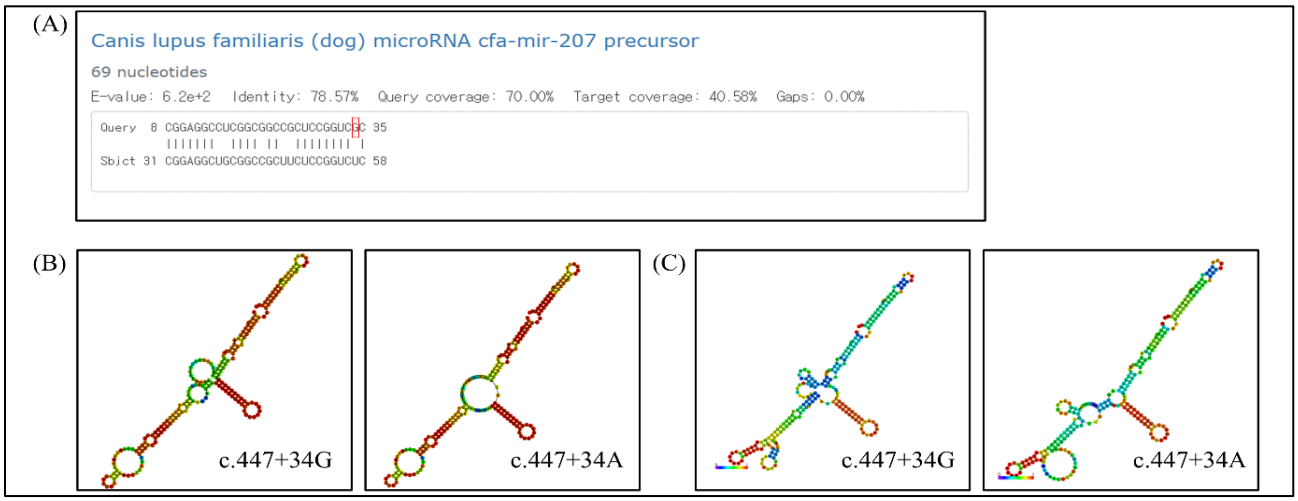

Fig. 4: Prediction results of *in silico* tools. (A) Result from miRBase. The red box represents the G allele of the c.447+34G>A SNP. (B) RNAfold-predicted secondary structures: c.447+34G (left) and c.447+34A (right). (C) CentroidFold-predicted secondary structures: c.447+34G (left) and c.447+34A (right).

Table 3: Linkage disequilibrium (LD) among polymorphisms of raccoon dog *IFITM3* gene.

	1	2	INDEL1	4	5	6	7	8	9	INDEL2	11	12	13	14	15	16	17
1	-																
2	0.029	-															
INDEL1	0.001	0.003	-														
4	0.365	0.024	0.008	-													
5	0.004	0.110	0.0	0.116	-												
6	0.276	0.008	0.0	0.159	0.008	-											
7	0.059	0.162	0.008	0.156	0.029	0.025	-										
8	0.012	0.404	0.001	0.013	0.110	0.003	0.065	-									
9	0.0	0.077	0.004	0.074	0.014	0.012	0.476	0.031	-								
INDEL2	0.132	0.170	0.011	0.210	0.043	0.036	0.744	0.096	0.354	-							
11	0.023	0.183	0.002	0.039	0.036	0.006	0.127	0.067	0.060	0.187	-						
12	0.001	0.055	0.029	0.040	0.016	0.014	0.067	0.019	0.132	0.085	0.352	-					
13	0.174	0.166	0.008	0.160	0.030	0.048	0.518	0.067	0.246	0.762	0.130	0.083	-				
14	0.022	0.075	0.046	0.030	0.016	0.005	0.174	0.025	0.083	0.166	0.406	0.291	0.178	-			
15	0.027	0.088	0.054	0.056	0.025	0.0	0.150	0.018	0.071	0.129	0.368	0.297	0.154	0.724	-		
16	0.013	0.056	0.001	0.022	0.001	0.003	0.070	0.001	0.033	0.103	0.388	0.149	0.072	0.210	0.099	-	
17	0.012	0.021	0.001	0.020	0.0	0.003	0.014	0.002	0.069	0.096	0.016	0.036	0.067	0.023	0.019	0.001	-

INDEL1 and INDEL2 indicate c.-67delA and c.430_435delCATGAC polymorphisms, respectively. The $r^2 > 0.333$ indicates strong LD.

Computational prediction of functional and structural impacts of the P18S variant in raccoon-dog *IFITM3*: We assessed the functional consequences of the non-synonymous SNP c.52C>T (P18S) in raccoon-dog *IFITM3* using computational algorithms (Table 5). PolyPhen-2 and SIFT predicted the variant to be probably damaging (score: 0.958) and deleterious (score: 0.0), suggesting potential protein function disruption. In contrast, E-SNPs&GO classified the variant as benign (score: 0.2). Aggregation propensity analysis via AMYCO indicated low aggregation risk (score: 0.0). At the same time, SODA predicted reduced solubility for the P18S variant compared to the wild-type protein (score: -3.073).

Table 4: Comparison of haplotype frequencies of *IFITM3* polymorphisms between healthy controls and SFTS virus (SFTSV)-infected raccoon dogs.

Haplotype	Controls (n=130)	Cases (n=48)	P-value
GGITGCTCCDCGATGGC	0.342	0.286	0.475
GGITGCCCTICGGTGGC	0.090	0.042	0.286
GGITGCCCCDCGGTGGC	0.058	0.065	0.880
GGITGCCCTICGGTGGT	0.064	0.021	0.256
Others	0.446	0.586	N/A

Others contain rare haplotypes with frequency <0.05. D and I indicate deletion and insertion, respectively.

Table 5: *In silico* programs evaluation on the effect of the non-synonymous single nucleotide polymorphism (SNP) in the raccoon dog.

	Program	Score	Prediction
c.52C>T (P18S)	PolyPhen-2	0.958	Probably damaging
	SIFT	0	deleterious
	E-SNPs&GO	0.21	Benign
	AMYCO	0	Low aggregation
	SODA	-3.073	Less soluble

Prediction of structural alteration in raccoon dog *IFITM3* induced by the P18S variant: Lastly, we explored the effects of a non-synonymous SNP (P18S) on the 3D structure of the raccoon dog *IFITM3* protein (Fig. 5). First, AlphaFold2 predicted the 3D structures of wild-type raccoon dog *IFITM3*. Then, the predicted structure was imaged using Swiss-PdbViewer, and the structural differences between the wild-type and variant proteins were analyzed. The hydrogen bond was absent in the P18 allele, but the S18 allele showed two hydrogen bonds with T19 (2.40 Å and 3.27 Å).

DISCUSSION

Recent developments in genomics have shed light on how genetic components contribute to susceptibility to a range of infectious diseases. The *IFITM3* protein has been linked to susceptibility to multiple viral infections, including West Nile virus, dengue virus, rhinovirus, coronavirus, human immunodeficiency virus, influenza A virus, and SFTSV infection (Brass *et al.*, 2009; Lu *et al.*, 2011; Xing *et al.*, 2020; Du *et al.*, 2024). While the *IFITM3* gene could be a significant candidate for SFTSV infection, no studies have examined genetic variation in raccoon dogs, a potential host for direct transmission of SFTS to humans. This species is of particular concern as its population increases. It inhabits both mountainous and urban areas in Korea, raising public health alerts about its potential as a vector for zoonotic diseases such as SFTS (Hong *et al*., 2013; Choi *et al*., 2024). This study explored the genetic characterization and polymorphisms of the raccoon dog *IFITM3* gene. The association between healthy and SFTSV-infected raccoon dogs has been further investigated. We identified 17 novel polymorphisms of the raccoon dog *IFITM3* gene. Only one SNP showed a noteworthy positive correlation between healthy and SFTSV-infected raccoon dogs.

The *IFITM3* protein is a member of the *IFITM* protein family, which are membrane-associated proteins that directly interfere with virus entry into the host's cells (Siegrist *et al.*, 2011). *IFITM* proteins restrict infection by many enveloped or non-enveloped viruses (Perreira *et al.*, 2013; Bailey *et al.*, 2014). In a previous study, human *IFITM3* has been shown to restrict SFTSV infection, especially by inhibiting viral entry (Du *et al.*, 2024). However, to date, there are few studies on the *IFITM3* protein in SFTSV-infected raccoon dogs.

In the present study, we first investigated the polymorphisms of the raccoon dog *IFITM3* gene (Fig. 1). We identified 17 novel polymorphisms in the raccoon dog *IFITM3* gene. We compared genotypes, alleles, and haplotype frequencies of the *IFITM3* gene between healthy and SFTSV-infected dogs. Notably, there was an association between susceptibility to SFTSV infection and *IFITM3* polymorphism (c.447+34G>A) (Table 2). This SNP is located within the 3′ untranslated region (3′ UTR) of the *IFITM3* gene. It is similar to the dog microRNA cfa-mir-207 precursor (Gene ID: 100886181) (Fig. 4A). The SNPs in 3′ UTR may influence the affinity of certain microRNAs, thereby regulating gene expression (Li *et al.*, 2020; Rykova *et al.*, 2022). Furthermore, the predicted RNA structures of the polymorphism alleles are illustrated in Fig. 4B and C. MicroRNAs exert their regulatory effect on target mRNAs primarily by promoting translational repression and subsequent mRNA degradation (Huntzinger and Izaurralde, 2011). We found not only variations in structure but also corresponding changes in the minimum free energy (MFE). MFE is applied for the detection of microRNA precursors and targets (Lorenz *et al.*, 2011; Uddin *et al.*, 2015).

Our findings suggest that *IFITM3* SNP (c.447+34G>A) can contribute to the structural diversity of RNA. Because c.447+34G>A is located in the 3' untranslated region (UTR), this substitution could affect RNA folding and the accessibility of microRNA binding sites. Changes in miRNA affinity may change *IFITM3* mRNA stability or translational efficiency. Considering that *IFITM3* is a key antiviral restriction factor that inhibits viral membrane fusion, such regulatory changes could affect the host's ability to limit SFTSV invasion and early replication *in vivo*. This prediction suggests further research is needed to see if the identified SNP affects *IFITM3* protein expression. This study is highly exploratory due to the lack of prior research on the *IFITM3* gene in raccoon dogs. The absence of *in vitro* or *in vivo* functional analyses remains a limitation of this study. Therefore, our primary goal was to identify genetic variation rather than experimentally validate the functional consequences of each SNP. It's important to confirm the biological effects of SNPs, especially since this study currently lacks further experimental validation (wet-lab confirmation).

Although a specific genetic variant, c.447+34G>A, initially seemed to be associated with SFTSV ($P<0.05$), this significant association disappeared after applying the strict

Supplementary Table 1: The genotype and allele frequencies of the *IFITM3* polymorphisms between healthy controls and SFTS virus (SFTSV)-infected raccoon dogs.

Variants		Genotype frequency, n (%)			P-value	Allele frequency, n (%)		P-value	HWE
		GG	GA	AA		G	A		
c.-251G>A	Controls	51 (0.78)	14 (0.22)	0	0.778	116 (0.89)	14 (0.1)	0.791	0.3305
	Cases	18 (0.75)	6 (0.25)	0		42 (0.88)	6 (0.12)		0.4840
		GG	GT	TT		G	T		
c.-179G>T	Controls	42 (0.65)	22 (0.34)	1 (0.01)	0.858	106 (0.82)	24 (0.18)	1	0.3166
	Cases	15 (0.63)	9 (0.37)	0		39 (0.81)	9 (0.19)		0.2582
		wt/wt	wt/del	del/del		wt	del		
c.-67delA	Controls	63 (0.97)	2 (0.03)	0	1	128 (0.98)	2 (0.02)	1	0.8997
	Cases	24 (1)	0	0		48 (1)	0		-
		TT	TC	CC		T	C		
c.-37T>C	Controls	45 (0.69)	19 (0.29)	1 (0.01)	0.409	109 (0.84)	21 (0.16)	0.379	0.5237
	Cases	14 (0.58)	9 (0.37)	1 (0.04)		37 (0.77)	11 (0.23)		0.7634
		GG	GT	TT		G	T		
c.-32G>T	Controls	60 (0.92)	5 (0.08)	0	1	125 (0.96)	5 (0.04)	1	0.7470
	Cases	22 (0.92)	2 (0.08)	0		46 (0.96)	2 (0.04)		0.8313
		CC	CA	AA		C	A		
c.-27C>A	Controls	60 (0.92)	5 (0.08)	0	1	126 (0.97)	4 (0.03)	1	0.7470
	Cases	23 (0.96)	1 (0.04)	0		47 (0.98)	1 (0.02)		0.9169
		CC	CT	TT		C	T		
c.52C>T (P18S)	Controls	11 (0.17)	54 (0.83)	0	1	76 (0.58)	54 (0.42)	1	0.0000
	Cases	4 (0.17)	20 (0.83)	0		28 (0.58)	20 (0.42)		0.0004
		CC	CT	TT		C	T		
c.147C>T	Controls	53 (0.82)	12 (0.18)	0	0.751	117 (0.90)	13 (0.10)	0.563	0.4122
	Cases	21 (0.88)	3 (0.12)	0		45 (0.94)	3 (0.06)		0.7439
		CC	CT	TT		C	T		
c.399C>T	Controls	30 (0.46)	35 (0.54)	0	0.347	95 (0.73)	35 (0.27)	0.329	0.0029
	Cases	14 (0.58)	10 (0.42)	0		39 (0.81)	9 (0.19)		0.1973
		wt/wt	wt/del	del/del		wt	del		
c.430_435delCATGAC	Controls	1 (0.02)	64 (0.98)	0	0.469	66 (0.51)	64 (0.49)	1	0.0000
	Cases	1 (0.04)	23 (0.96)	0		25 (0.52)	23 (0.48)		0.0000
		CC	CT	TT		C	T		
c.447+29C>T	Controls	45 (0.69)	20 (0.31)	0	1	110 (0.85)	20 (0.15)	1	0.1426
	Cases	17 (0.71)	7 (0.29)	0		41 (0.85)	7 (0.15)		0.4029
		GG	GA	AA		G	A		
c.447+34G>A	Controls	33 (0.51)	29 (0.45)	3 (0.04)	P<0.05	95 (0.73)	35 (0.27)	0.577	0.2805
	Cases	14 (0.58)	5 (0.21)	5 (0.21)		33 (0.68)	15 (0.31)		0.0116
		GG	GA	AA		G	A		
c.447+124G>A	Controls	10 (0.15)	55 (0.85)	0	1	75 (0.58)	55 (0.42)	1	0.0000
	Cases	4 (0.17)	20 (0.83)	0		28 (0.58)	20 (0.42)		0.0004
		TT	TC	CC		T	C		
c.447+146T>C	Controls	39 (0.60)	25 (0.38)	1 (0.01)	0.267	103 (0.79)	27 (0.21)	0.672	0.1739
	Cases	17 (0.71)	6 (0.25)	1 (0.04)		40 (0.83)	8 (0.17)		0.6242
		GG	GC	CC		G	C		
c.447+151G>C	Controls	43 (0.66)	21 (0.32)	1 (0.01)	0.473	107 (0.82)	23 (0.18)	1	0.3781
	Cases	17 (0.71)	6 (0.25)	1 (0.04)		40 (0.83)	8 (0.17)		0.6242
		GG	GA	AA		G	A		
c.447+180G>A	Controls	53 (0.82)	12 (0.18)	0	1	118 (0.91)	12 (0.09)	1	0.4122
	Cases	20 (0.83)	4 (0.17)	0		44 (0.92)	4 (0.08)		0.6560
		CC	CT	TT		C	T		
c.447+216C>T	Controls	53 (0.82)	12 (0.18)	0	0.751	118 (0.91)	12 (0.09)	0.762	0.4122
	Cases	21 (0.88)	3 (0.12)	0		45 (0.94)	3 (0.06)		0.7439

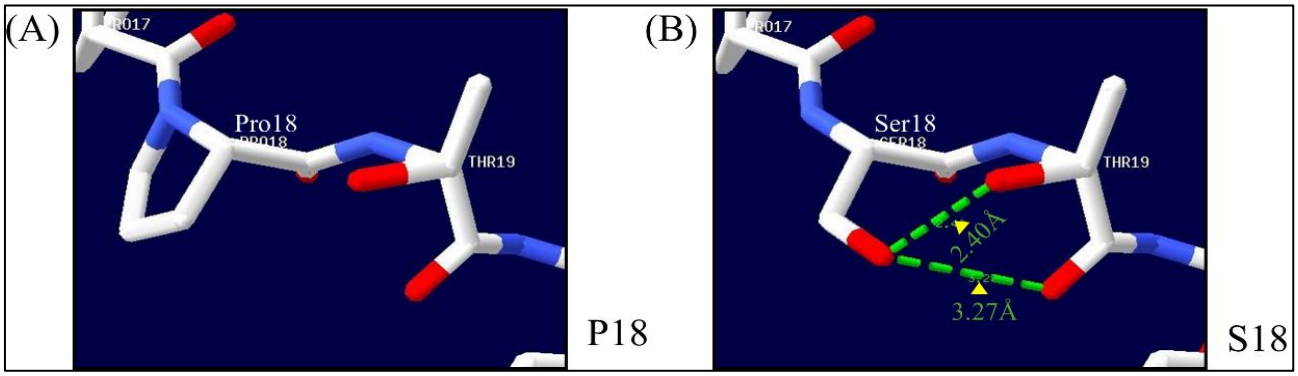

Fig. 5: The 3D structure and hydrogen bonds of *IFITM3* protein in raccoon dogs. The left indicates the 3D structure with the wild-type allele, and the right indicates the 3D structure with the non-synonymous SNP. The yellow arrows represent changes in hydrogen bonds.

statistical adjustment known as the Bonferroni correction (the adjusted P=0.336). Therefore, to definitively determine the role of this genetic variant in disease susceptibility, larger and more extensive follow-up studies are necessary.

A coordinated role in controlling the *IFITM3* gene is implied by LD and haplotype blocks. The distinctive haplotype patterns are therefore important because they probably influence the expression of the *IFITM3* gene through complex genetic interactions, identifying particular genomic areas that require in-depth functional analysis.

IFITM3 possesses the endocytosis YXXΦ motif (Blyth *et al.*, 2016). The YXXΦ motif is also found in the raccoon dog *IFITM3* gene (Fig. 3). The YXXΦ motif constitutes part of a distinct type of signaling motif, which regulates immune responses through activating and inhibitory receptors on the surface of immune and other cells (Kakkanas *et al.*, 2022). Some viruses have incorporated the YXXΦ motif into their proteins and use it for immune regulation.

Next, we estimated the effects of amino acid substitutions on the raccoon dog *IFITM3* protein using *in silico* programs. PolyPhen-2 and SIFT predicted that the amino acid substitution (P18S) was deleterious (Table 5). The P18S variant may negatively affect *IFITM3* protein function and stability. To determine its impact, future research should employ site-directed mutagenesis to generate P18S mutant proteins and investigate their ability to restrict SFTSV entry in cell culture models.

It is important to note that SFTSV can cause asymptomatic infections (Wang *et al.*, 2014), which may confound the understanding of the role of *IFITM3* in virus infection. Given that *IFITM3* limits viral entry, its effectiveness in preventing asymptomatic infections of SFTSV remains an important area for further research, particularly in understanding how it may influence the spread of the virus in raccoon dogs. The limitation of this study includes the relatively small cohort size. Future research involving larger cohorts would be highly beneficial for confirming these findings and enhancing the reliability of the conclusions reached from this study. Due to the raccoon dog's diverse habitats in Korea, an unrecognized population substructure or sampling bias from limited geographical collection may affect allele frequencies. Therefore, larger, geographically stratified studies are essential to validate the genetic associations found in this study.

Conclusions: Our study focused on examining *IFITM3* polymorphisms in both SFTSV-infected and healthy raccoon dogs. We found that there's a notable connection between a specific *IFITM3* SNP (c.447+34G>A) and how susceptible an animal is to SFTSV infection. This particular SNP (c.447+34G>A) is thought to impact RNA structure and energy, which could, in turn, change how genes are regulated. While these results suggest an initial association, not direct causation, between *IFITM3* variants and SFTSV susceptibility, they clearly highlight the need for more in-depth functional studies. Moreover, these findings emphasize the critical role of continuous zoonotic monitoring in wild carnivores, especially in species like the raccoon dog.

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