VR **VETERINARY RESEARCH**

Identification and genotyping of feline infectious peritonitis-associated single nucleotide polymorphisms in the feline interferon-γ gene

Li-En Hsieh and Ling-Ling Chueh[*]

Abstract

Feline infectious peritonitis (FIP) is an immune-mediated, highly lethal disease caused by feline coronavirus (FCoV) infection. Currently, no protective vaccine or effective treatment for the disease is available. Studies have found that some cats survive the challenge of virulent FCoV isolates. Since cellular immunity is thought to be critical in preventing FIP and because diseased cats often show a significant decrease in interferon-γ (IFN-γ) production, we investigated whether single nucleotide polymorphisms (SNP) in the feline IFN-γ gene (*flFNG*) are associated with the outcome of infection. A total of 82 asymptomatic and 63 FIP cats were analyzed, and 16 SNP were identified in intron 1 of *flFNG*. Among these SNP, the *fFING + 428 T* allele was shown to be a FIP-resistant allele ($p = 0.03$), and the heterozygous genotypes *01C/T* and *+408C/T* were found to be FIP-susceptible factors ($p = 0.004$). Furthermore, an *flFNG + 428* resistant allele also showed a clear correlation with the plasma level of IFN-γ in FIP cats. For the identification of these three FIP-related SNP, genotyping methods were established using amplification refractory mutation system PCR (ARMS-PCR) and restriction fragment length polymorphisms (RFLP), and the different genotypes could easily be identified without sequencing. The identification of additional FIP-related SNP will allow the selection of resistant cats and decrease the morbidity of the cat population to FIP.

Introduction

Feline infectious peritonitis (FIP) is an immune-mediated disease caused by feline coronavirus (FCoV) infection. Despite the vast number of studies conducted since the recognition of FIP [1], neither effective vaccines nor therapeutic agents are available for the prevention or treatment of this often fatal disease. Therefore, it remains one of the most important feline infectious diseases.

Despite the ubiquitous existence of FCoV in cat populations around the world, the development of FIP was only observed in fewer than 5% of the FCoV-infected cats [2]. In addition, during the past few decades, several studies were conducted using various FCoV isolates, and regardless of the dose of virulent FCoV, some cats survived from the experimental infection [2-11]. Genetic polymorphisms in the host were suggested to be important in the resistance to FIP [2]; however, no specific gene

with a clear correlation to resistance to this disease has ever been identified.

The protective immunity to FIP is thought to result mainly from cell-mediated immunity (CMI), and changes in the expression of several cytokines have been observed in cats with either experimentally induced or naturally occurring FIP [2]. The expression of one of the cytokines studied, interferon-γ (IFN-γ), was consistently decreased in diseased animals, and this gene is thought to play a protective role in the pathogenesis of FIP, since it is a key cytokine in CMI [6,8,12-14].

In recent decades, single nucleotide polymorphisms (SNP) in the IFN-γ gene (*IFNG*) have been found to be associated with various pathological conditions in humans [15] as well as in ruminants and fowl [16-23]. Nevertheless, this gene has not been investigated in cats. To identify a possible association between feline *IFNG* (*flFNG*) SNP and the outcome of FCoV infection, some regions of the *flFNG* gene were sequenced and analyzed. Three SNP with statistical relevance were found to be associated with the occurrence of FIP,

* Correspondence: linglingchueh@ntu.edu.tw
Graduate Institute of Veterinary Medicine, School of Veterinary Medicine, National Taiwan University, Taipei 10617, Taiwan

and polymerase chain reaction (PCR) assays based on these differences were designed as a potential screening test for the selection of FIP-resistant populations.

Materials and methods
Specimens
Whole blood and buccal swabs were collected from 82 FCoV-infected asymptomatic cats and 64 FIP cats from 2005 to 2012 at the National Taiwan University Animal Hospital for the association analysis. All asymptomatic healthy cats were three years old or younger and had positive reverse transcription-nested PCR test results for FCoV infection [24] in any of the following samples upon first arriving at the hospital: whole blood or nasal, oral, conjunctival, or rectal swabs. In addition, these cats showed no FIP-related symptoms when recruited into the study, and except for 12 cats, they stayed healthy for at least two years. All the FIP cases enrolled in this study were cats showing typical clinical signs of FIP and further confirmed by necropsy, histopathological examinations and FCoV detection [24] in disease-associated tissues, i.e. body effusions, kidney, liver, spleen, mesenteric lymph node, lung, and/or brain.

To elucidate the role of host genetic background in the development of FIP, two viral pathogens, i.e. feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), that cause immunosuppression in cats were checked in FIP cats using nested PCR [25,26], and the positive cats were omitted from the association analysis.

Identification of SNP in the partial fIFNG sequences
The genomic DNA from each cat was extracted using a genomic DNA mini kit (Geneaid Biotech, New Taipei City, Taiwan), and partial *fIFNG* sequences were amplified by PCR. Briefly, the genomic DNA was amplified with primers aligning to either the 5′ proximal regulatory region and intron 1 or the 5′ untranslated region and exon 2 of *fIFNG* (Table 1), and the PCR products were then sequenced from both ends using an auto sequencer ABI 3730XL (Applied Biosystems, San Mateo, USA). The sequences were aligned by Geneious 3.8.5 (Biomatters, Auckland, New Zealand), and the polymorphisms were identified.

Linkage disequilibrium (LD) test and association analysis
An LD test and the creation of an LD plot were performed using $LD_2SNPing$ v 2.0 (Department of Electronics Engineering, National Kaohsiung University of Applied Science, Kaohsiung, Taiwan) [27]. The associations between SNP and the outcome of FCoV infection were analyzed. A Fisher's exact test value of $P < 0.05$ was considered to represent a significant association.

Table 1 Primers used for the identification of SNP, ARMS-PCR and RFLP

Primer	Orientation[a]	Position[b]	Sequence (5′ - 3′)[c]	T_A[d]	Amplicon size
Identification of SNP					
5′-PRR[e] - intron 1	F	−677 ~ -654	CAGGGCAATGCAAAGCTGTGGTAG	65°C	1306 bp
	R	+629 ~ +607	GCGGCAGTAGAACTTTGAAACCA		
5′-UTR[f] - exon 2	F	−44 ~ -25	CGGAGCTACTGATTTCAACT	63°C	1434 bp
	R	+1390 ~ +1371	GGAAAGAGGTAAGCTGGGTA		
Genotyping[g]					
+401	F (universal)	+320 ~ +341	GGGGCATTCATCAGTCTTCCAG	56°C	200 bp
	R (universal)	+519 ~ +500	AAGGTCAGGGTTAGCATGAA		
	F (*T* allele)	+382 ~ +402	TAATTTTGTGGTGAGAATC*T*A		138 bp
	R (*C* allele)	+418 ~ +400	CAACATCACAGTCTAATG*G*		99 bp
+408	F	+320 ~ +341	GGGGCATTCATCAGTCTTCCAG	56°C	200 bp
	R	+519 ~ +500	AAGGTCAGGGTTAGCATGAA		
+428	F (universal)	+288 ~ +307	TACCCTCTGCTCAACTTGCT	67°C	232 bp
	R (universal)	+519 ~ +500	AAGGTCAGGGTTAGCATGAA		
	F (*T* allele)	+408 ~ +429	CTGTGATGTTGGGTAGTGTG*T*C		112 bp
	R (*C* allele)	+449 ~ +427	GGCTAGTCATTGTTTCAATAG*G*C		162 bp

[a]*F*: forward; *R*: reverse.
[b]The nucleotide positions start from the first translation start point (+1).
[c]The italicizing indicates the target SNP.
[d]Annealing temperature.
[e]5′-proximal regulatory region.
[f]5′-untranslated region.
[g]PCR was done with a heating and cooling rate of 3 and 2 °C, respectively.

Quantification of IFN-γ levels in the plasma samples of FIP cats

The plasma samples of FIP cats collected at the day of presentation were stored at −20 °C before use. The concentration of IFN-γ in the plasma samples was determined using an antigen capture ELISA (R & D system, McKinley Place NE, USA) following the procedure advised by the manufacturer's instructions.

Genotyping of SNP by tetra-primer amplification refractory mutation system PCR (ARMS-PCR) and restriction fragment length polymorphisms (RFLP)

For the genotyping of SNP using ARMS-PCR, the PCR reactions contained 1 μL of template DNA, each primer at 500 nM, 200 μM dNTP, 1.5 mM MgCl$_2$, and 0.4 U Phusion DNA polymerase (Thermo Scientific, Waltham, USA) in a total volume of 20 μL with 1× Phusion HF buffer. The primers and PCR conditions are listed in Table 1. The PCR products were resolved in 2% agarose gels and photographed using an imaging system. The PCR for the genotyping of SNP with RFLP was carried out with the same protocol, and the PCR products were

digested with *HpyCH4*III (New England Biolabs, Ipswich, USA) following the manufacturer's instructions. The digested DNA was resolved in 2% agarose gels and photographed using an imaging system.

Results

Polymorphisms in fIFNG

Due to a lack of information on polymorphisms in *fIFNG*, DNA from 40 cats, including 20 asymptomatic and 20 FIP cats, was sequenced throughout the proximal regulatory region, the 5′-UTR, exon 1, intron 1 and a partial exon 2 region of *fIFNG*. In the 2067 bp analyzed, 3 repeat regions and 16 SNP were identified (Figure 1A). Among these 19 polymorphisms, only one was located in the proximal regulatory region, and the others were in intron 1. No polymorphisms were identified in the 5′-UTR, exon 1 or exon 2. Intron 1 was more polymorphic than the upstream region of *fIFNG*. The allele frequencies of all the SNP surveyed were identified and are listed in Figure 1. The mean allele frequencies of the minor alleles ranged from 4.8% to 47.6%.
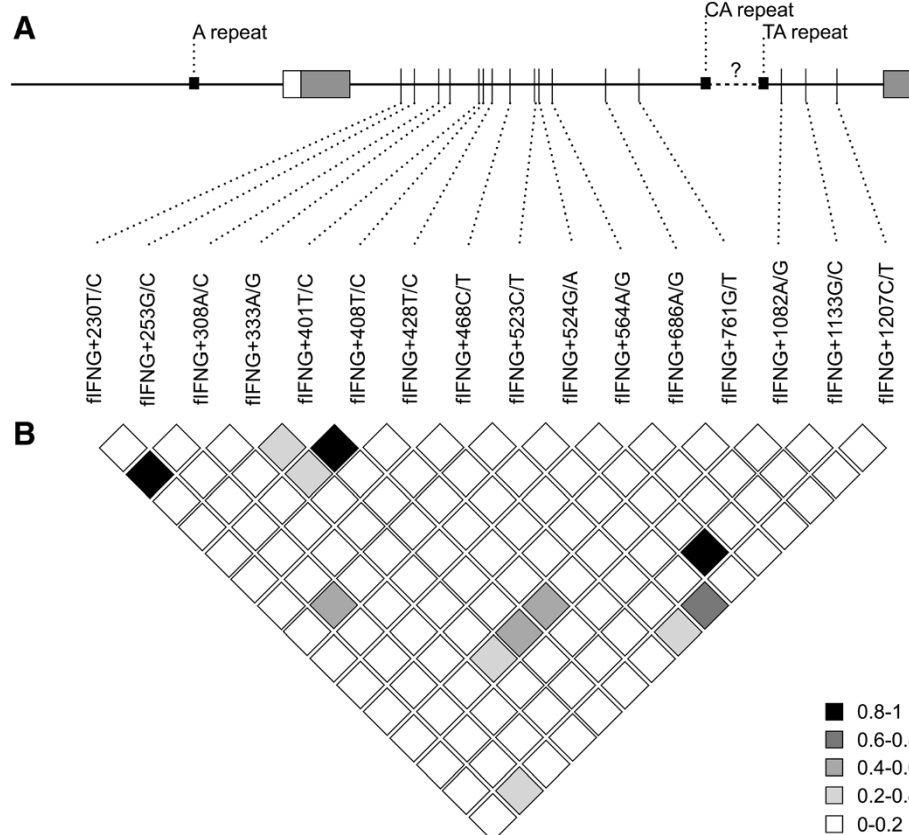

Figure 1 A schematic of the partial *fIFNG* sequences analyzed in this study. (A) A partial *fIFNG* sequence of 2067 bp was sequenced in this study, including the 5′ proximal regulatory region, 5′ UTR, exon 1, intron 1 and a partial exon 2 region. Black box: repeat region. White box: 5′ UTR. Gray boxes: exons 1 and 2. ?: a ~115 bp region between 2 repeat regions of unknown sequence. **(B)** LD plot of the 16 SNP tested in this study. The r^2 values between different SNP are indicated by different colors.

The SNP were then subjected to LD analysis. An LD plot of all the SNP for the whole population, including asymptomatic and FIP cats, was generated (Figure 1B). Three pairs of SNP were found to be significantly associated ($r^2 > 0.8$): *fIFNG + 230 T/C* and *fIFNG + 308A/C*, *fIFNG + 401 T/C* and *fIFNG + 408 T/C*, and *fIFNG + 524G/A* and *fIFNG + 1133G/C*. *fIFNG + 230* and *fIFNG + 308* and *fIFNG + 401* and *fIFNG + 408* had a high degree of LD and were inherited together 100% of the time.

Association study

To elucidate the association between the identified SNP and the outcome of FCoV infection, the frequency of each genotype (Additional file 1) and allele (Additional file 2) was analyzed in 82 asymptomatic and 63 FIV and FeLV-free FIP cats. From all the SNP tested, only *fIFNG + 428C/T* was found to be significantly associated with the outcome of the infection. At position +428, there was a higher frequency of the *CT* genotype in asymptomatic control cats (19.5%) than in FIP cats (6.3%), and the data showed a significant correlation with disease resistance ($p = 0.03$) (Table 2). Similarly, the analysis of allele frequency and disease outcome also revealed that the *T* allele at position +428 was significantly associated with resistance to FIP ($p = 0.03$) (Table 2).

Although both types I and II FCoV can cause FIP, type II FCoV has been found to be more related to acute infection [28] and can cause horizontal transmission [29] whereas infection with type I viruses often results in persistent infection [30], therefore the host genotype involved in type I FCoV infection likely influences the resistance to a greater extent. To gain a better insight into the effect of host genetic variation in *fIFNG* and FIP, 29 type I FCoV-infected FIP cats were selected for further analysis (Additional files 3 and 4). After analyzing the target population, despite no allele of any SNP showing an association with the infection outcome (Table 3), the heterozygous genotype (*CT* genotype) at positions +401 and +408 were found to be significantly associated with susceptibility to FIP ($p = 0.004$) (Table 3).

Table 2 FIP-associated SNP in *fIFNG* identified in the whole cat population in this study

SNP	Control number (%)	FIP number (%)	OR (95% CI)	P
fIFNG + 428				
CC	66 (80.5)	60 (93.8)	3.6 (1.2-11.5)	0.03
CT	16 (19.5)	4 (6.3)	Reference	
TT	0 (0.0)	0 (0.0)	…[a]	
Allele C	148 (90.2)	124 (96.9)	3.4 (1.1-10.3)	0.03
Allele T	16 (9.8)	4 (3.1)	Reference	

[a]no OR can be calculated.

Table 3 FIP-associated SNP in *fIFNG* identified in the type I FCoV-infected cats

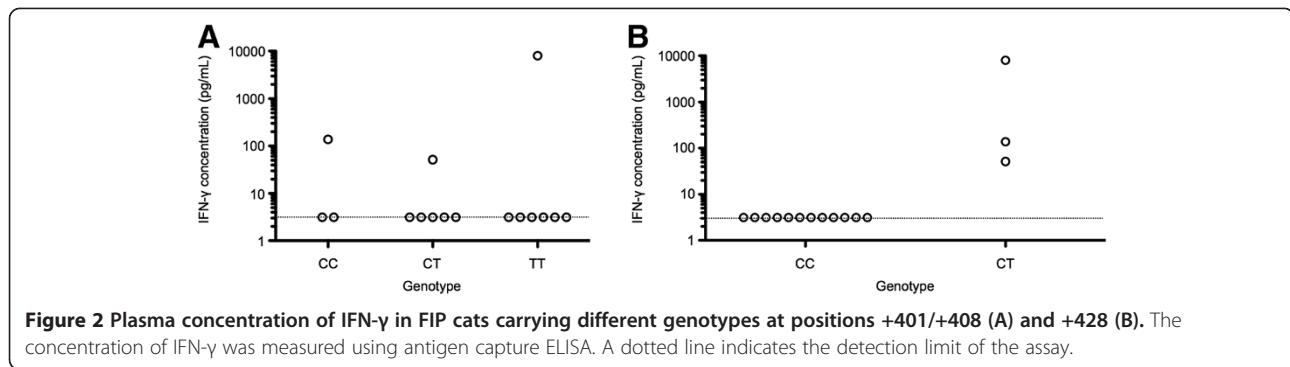| SNP	Control number (%)	FIP number (%)	OR (95% CI)	P
fIFNG + 401				
TT	43 (52.4)	11 (37.9)	0.4 (0.2-0.9)	0.004
CT	26 (31.7)	18 (62.1)	Reference	
CC	13 (15.9)	0 (0.0)	n/a[a]	
Allele C	112 (68.3)	40 (69.0)	…[b]	1.00
Allele T	52 (31.7)	18 (31.0)	…	
fIFNG + 408				
TT	43 (52.4)	11 (37.9)	0.4 (0.2-0.9)	0.004
CT	26 (31.7)	18 (62.1)	Reference	
CC	13 (15.9)	0 (0.0)	n/a[a]	
Allele C	112 (68.3)	40 (69.0)	…	1.00
Allele T	52 (31.7)	18 (31.0)	…	

[a]not available.
[b]no OR can be calculated.

Production of IFN-γ in FIP cats carrying different genotypes

To further validate the correlation between FIP-associated SNP and the production of IFN-γ, the concentration of IFN-γ in the plasma samples from 15 FIP cats was measured. For most of the FIP cats (12/15; 80%), the concentration was below the detection limit (<3.125 pg/mL) except for three cats. The concentrations of IFN-γ for cat 14, 17, and 69 were 52, 138 and > 8000 pg/mL, respectively (Figure 2, Additional file 5). These three animals were the only cats carrying the resistant allele (*T allele*) at position +428 whereas others were *fIFNG + 428CC* genotype (Figure 2B, Additional file 5). For the *fIFNG + 401/+408*, however, no clear correlation between the IFN-γ responses and genotypes was observed (Figure 2A, Additional file 5).

Genotyping for disease-related SNP

Through the association analysis and the quantification of plasma IFN-γ level, *fIFNG + 428C/T* was found to be associated with FIP and the plasma concentration of IFN-γ. In addition to this SNP, *fIFNG + 401C/T* and *fIFNG + 408C/T* were also shown to be associated with the type I FCoV-infected FIP population. To develop screening that can easily distinguish the resistant cats from the at-risk ones, ARMS-PCR specific for *fIFNG + 428C/T* and *fIFNG + 401C/T* was attempted. Using the tetra primer ARMS-PCR, the different alleles at position +401 could be successfully distinguished: universal control product: 200 bp; *T* allele: 138 bp; *C* allele: 99 bp (Figure 3A). Similarly, the *C* and *T* alleles could be determined at position +428: universal control product: 232 bp; *C* allele: 162 bp; *T* allele: 112 bp (Figure 3C). For *fIFNG + 408*,

Figure 2 Plasma concentration of IFN-γ in FIP cats carrying different genotypes at positions +401/+408 (A) and +428 (B). The concentration of IFN-γ was measured using antigen capture ELISA. A dotted line indicates the detection limit of the assay.

RFLP was used for genotyping, and the digested PCR products for the *T* allele could be found at 151 bp and 54 bp; the digested PCR products for the *C* allele could be found at 113 bp, 54 bp, and 43 bp (Figure 3B).

Discussion

The occurrence of FIP is thought to be largely affected by viral virulence factors, and their roles in FIP have been intensely studied. Several viral genes, including *spike* [31-33], accessory gene *3c* [34-36] and *7b* [37-39], and *membrane* [40], have been proposed to play important roles in the development of the disease in FCoV-infected cats. As a disease with immunopathogenesis entity, the knowledge of the host genetic factors that affect FIP is still very limited, and only two studies have reported such factors [41,42], in contrast to the considerable quantity of information on the virulence factors.

Addie et al. who conducted the first study, attempted to reveal the genetic background of the cats and their associations with the occurrence of FIP (Table 4) [42]. Considering its important role in the defense against viral infection, *feline leucocyte antigen (FLA)-DRB* polymorphisms were analyzed in four different domestic shorthaired (DSH) or other purebred populations: FIP, FCoV carrier, transient infection and resistant. However, no association was found between any of the *FLA-DRB* polymorphisms and the

occurrence of FIP, which might be due to the small sample size (FIP: 8 cats; asymptomatic: 17 cats) used. To gain better insight, a larger number of cats in each population was used (FIP: 63 cats; asymptomatic: 82 cats), and the association was clearly observed in the present study (Table 4).

Recently, several FIP-associated SNP were identified by a massive screen of Birman cats using a commercialized SNP gene chip, and the candidate genes were located from 69 kb to more than 1 Mb away from the identified SNP (Table 4) [41]. In contrast to that report, the SNP were located within the candidate gene, *fIFNG*, in our study, which demonstrates a stronger linkage of the analyzed SNP to the candidate gene. In addition, the cat populations enrolled in this study, including approximately 50% DSH and another 50% pure breed or mixed breed cats, were more variable, and the SNP associated with the disease may have been better represented on the entire cat population.

IFN-γ is a crucial regulatory cytokine in CMI and is important for the control of intracellular pathogens. In FCoV infection, decreased IFN-γ production in FIP cats was consistently observed in multiple studies [6,8,12-14]. In addition, in FCoV-infected non-FIP cats, the peripheral blood mononuclear cells showed a significant increase in the IFN-γ response upon stimulation with the FCoV antigen when compared to the FIP cats [3]. The expression of
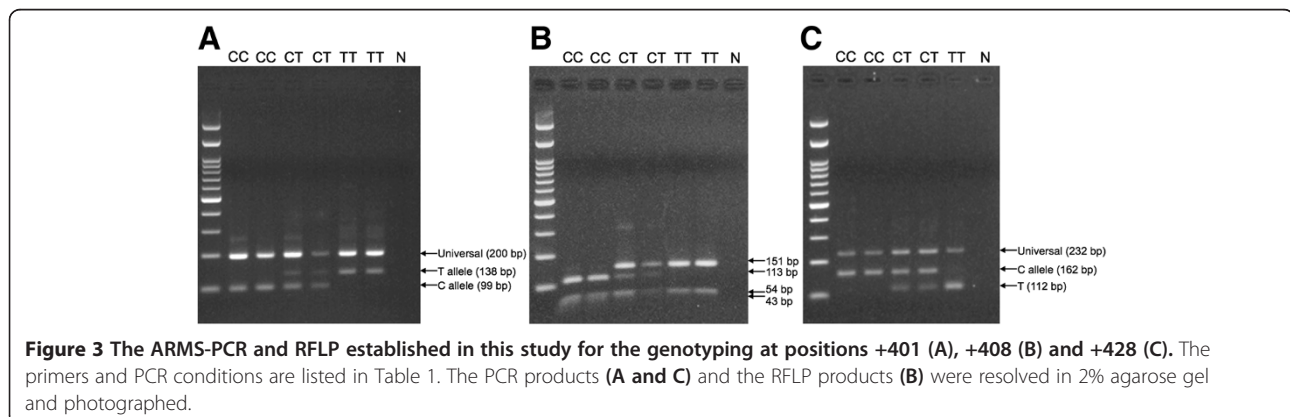

Figure 3 The ARMS-PCR and RFLP established in this study for the genotyping at positions +401 (A), +408 (B) and +428 (C). The primers and PCR conditions are listed in Table 1. The PCR products **(A and C)** and the RFLP products **(B)** were resolved in 2% agarose gel and photographed.

Table 4 Studies conducted on host genetic polymorphisms and their associations with FIP

Target	Method	Breed	Grouping (number of cats)	Polymorphism associated with FIP	Reference
FLA-DRB	1. Clonal sequence analysis	DSH[b] and Pure breed	1. FIP (8)	Non	[42]
	2. RSCA[a]		2. Carrier (4)		
			3. Transient (10)		
			4. Resistant (3)		
Identified SNP	SNP gene chip	Birman cats	1. FIP (34)	1. A2.191286425 - *ELMO1*	[41]
			2. Healthy (151)	2. A1.196617776 - *ERAP1, 2*	
				3. A1.206840008 - *ERAP1, 2*	
				4. Un.59861682 - *ERAP1, 2*	
				5. E2.65509996 - UI [c]	
fIFNG	PCR and sequencing	DSH and Pure breed	1. FIP (63)	1. *fIFNG + 401*	This study
			2. Healthy (82)	2. *fIFNG + 408*	
				3. *fIFNG + 428*	

[a]Reference strand mediated conformational analysis.
[b]Domestic shorthaired.
[c]Unidentified.

IFN-γ was thought to protect against FCoV infection. In several studies on human viral infections, i.e., human immunodeficiency virus [43], hepatitis B and hepatitis C [44,45], a similar correlation between the magnitude of the IFN-γ response and disease manifestation was identified. The polymorphisms in *IFNG* were associated with the diseases caused by these viruses [46-48].

The SNP associated with FIP are located in the intron 1 region. Therefore, it is unlikely that they mediate the outcome of the infection directly by altering the function of IFN-γ. In humans, *IFNG* polymorphisms have been found that correlate with the expression of IFN-γ. In the promoter region, SNP at positions −183 and −155 were found to influence the binding of activating transcription factor-1 and the nuclear factor-associated T-cell site [49,50]. In addition, the number of *CA* microsatellite repeats was found to be tightly associated with the SNP at position +874 and to influence the production of IFN-γ by altering the binding activity of nuclear factor kappa-light-chain-enhancer of activated B cells [15]. In ewes, despite the polymorphisms in *INFG* at position -641, the microsatellite showed a significant effect on *IFNG* expression in the spleen, although the mechanism that mediates this effect remains unknown. In the present study, a clear correlation between the IFN-γ responses and the genotype at position +428 was observed (Figure 2B). The resistant *T* allele at position +428 may serve as a major factor to enhance the IFN-γ production upon infections of intracellular pathogens. Despite lacking correlation with the IFN-γ level, the SNP at positions +401 and +408 might still play a minor role in the alteration of the production of IFN-γ. Since the three FIP-related SNP identified in this study are located in a small cluster, it is possible that these polymorphisms alter the binding of one or more transcription

factors and work together to influence the immune response to FCoV infection. However, the actual mechanism for the altered expression of IFN-γ remains to be investigated.

In this study, three SNP in *fIFNG* were found to be associated with the outcome of FCoV infection. Using the ARMS-PCR and RFLP tests established, the three SNP could be distinguished in the ordinary diagnostic laboratory without sequencing, and cats bearing either the susceptible or resistant genotypes could be identified. Since the disease outcome is usually influenced by multiple host genes, other candidate genes, i.e., tumor necrosis factor-α [51,52], interleukin-12 [6] and CD209 [53], are currently being surveyed. A combination of all the FIP-related SNP into a single genotyping microarray will allow the selection of resistant cats before breeding and eventually decrease the morbidity of the cat population to FIP.

Additional files

Additional file 1: Frequencies of the various genotypes and their associations with the outcome of FCoV infection. The CT genotype at position +428 was significantly associated with the resistance of FIP.

Additional file 2: Frequencies of various alleles and their associations with the outcome of FCoV infection. The T allele at position +428 was significantly associated with the resistance of FIP.

Additional file 3: Frequencies of various genotypes and their associations with the outcomes of type I FCoV infection. The heterologous genotypes of *fIFNG + 401* and *fIFNG + 408* were associated with the susceptibility to FIP in type I FCoV-infected cats.

Additional file 4: Frequencies of various alleles and their associations with the outcomes of type I FCoV infection. No allele of the analyzed SNP was associated with the occurrence of FIP in type I FCoV-infected cats.

> **Additional file 5: Concentration of IFN-γ in the plasma samples of FIP cats carrying different genotypes at positions + 401, +408, and +428 on *fIFNG*.** All the FIP cats carrying the CT genotype at position +428 were positive for the detection of plasma IFN-γ.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LEH performed the sample preparation, *fIFNG* amplification and sequencing, measurement of the plasma concentration of IFN-γ, established genotyping methods and prepared the manuscript. LLC conceived the study, participated in the study design and coordination and contributed to the preparation of the manuscript. Both authors read and approved the final manuscript.

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