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Real-time PCR assay for rapid differentiation of *env*-based genotypes of feline leukemia virus

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Abstract

The envelope protein plays an essential role in the pathogenesis of feline leukemia virus (FeLV). Three categories of genotypes have been identified according to the sequence of the envelope gene (*env*). The relationship between *env*-based genotype and viral pathogenicity remains to be investigated. However, this effort is hampered by the fact that sequencing and phylogenetic analysis of *env* are expensive and time-consuming. In this study, we identified single-nucleotide polymorphisms (SNPs) that are located in the long terminal repeat but linked to *env*-based genotypes (G), I and III. Our real-time PCR assay included a primer containing the SNP sites and detected GI proviruses but not GIII proviruses. It will help rapid differentiation of the *env*-based genotypes of FeLV field isolates.

Key Words: Feline leukemia virus, genotype, real-time PCR

Feline leukemia virus (FeLV) belongs to the genus *Gammaretrovirus* in the family Retroviridae and causes diverse diseases including lymphoma, anemia, and immunodeficiency in domestic cats[4)]. Antigenemia or viremia (or both) are seen in some—but not all—infected cats[6)]. After infection, provirus is persistently integrated into the host genome[2)], and viral RNA is continually transcribed from the provirus[7)].

Although the pathogenesis of FeLV is not well understood, the envelope protein (Env) plays an essential role in this process[1)]. This protein attaches to the cell-surface receptor for viral entry in the cell and thus initiates the viral life cycle[22)]. Variation in the envelope gene (*env*) sequences are generated through genetic mutation

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or due to recomebination with endogenous retroviral sequences during viral replication[13,17]. Such changes in *env* affect viral receptor utilization, replication efficiency, and disease outcome[3,14].

A comprehensive phylogenetic analysis based on *env* sequences has revealed that FeLVs are classified into 3 distinct genotypes[21]. These *env*-based genotypes are not associated with conventional receptor subgroups determined by viral interference assays[11,16,18]. Rather, these genotypes reflect geographic distribution. For example, genotype (G) I predominates in Japan, but GII and III occur also as minor populations. In contrast, only GIII is distributed in Europe and North and South America[21]. The relationship between *env*-based genotype and viral pathogenicity remains to be investigated. However, this effort is hampered by the fact that sequencing and phylogenetic analysis of *env* are expensive and time-consuming and are difficult to be perform routinely in diagnostic laboratories.

Real-time PCR analysis supports rapid genotyping of viral pathogens[10,15]. This method typically uses genotype-specific primers or probes (or both) to detect genotype-specific nucleotide sequences. However, designing such genotype-specific primers and probes for FeLV *env* sequences is difficult because they are highly variable among strains[21]. In contrast, the sequence of the U3 region in the long terminal repeat (LTR) is relatively conserved among FeLV strains[8]. Because of its high conservation, the U3 region is used as a target in previously developed real-time PCR assays to determine proviral copy number in FeLV-infected cats[19,20].

Here we identified single-nucleotide polymorphisms (SNPs) in the U3 region of FeLV that were linked to *env*-based genotypes, I and III. Using these SNPs, we developed a SNP-based real-time PCR assay for *env*-based genotyping to differentiate between GI and GIII isolates of FeLV.

To obtain FeLV proviruses, EDTA-treated blood samples were collected from 12 outdoor cats that presented at 9 animal hospitals in Morioka City (Iwate Prefecture, Japan) from September 2013 through July 2014. We carefully explained the purpose of the present study to the owners, and the blood samples were collected with their permission. Peripheral blood leukocytes were isolated by incubating the blood samples with 2 volumes of 0.83% NH_4Cl solution. Genomic DNA was isolated from the leukocytes or the feline lymphoma cell line (FeLV3281, RIKEN BioResource Center, Ibaraki, Japan) by using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). All animal experiments were approved by the Animal Experiment Committee of Iwate University (protocol no. A201527).

We amplified a part of the U3–*gag* region from genomic DNA as described previously[12] with modifications. The reaction mixture for the first run contained 5 μL of 100 ng genomic DNA, 0.25 μL of Ex Taq (5 U/μL; Takara Bio, Shiga, Japan), 2.5 μL of Ex Taq Buffer (Takara Bio), 0.25 μL of the forward primer U3-F (1) and the reverse primer G-R (1) (10 μM each), 4 μL of dNTP mixture (2.5 mM each), and sufficient deionized water to bring the volume to 25 μL. The reaction mixture for the second run included 1 μL of the reaction mixture from the first run as a template and 0.25 μL of the forward primer U3-F (2) and the reverse primer G-R (2) (10 μM each), with the remaining reagents as described for the first run. The first and second runs used the same reaction conditions: initial denaturation at 94°C for 60 sec; 55 amplification cycles comprising denaturation at 95°C for 45 sec, annealing at 65°C for 30 sec, and extension at 72°C for 60 sec; and a final extension step at 72 °C for 180 sec. All PCR primers were synthesized by Eurofins Genomics (Tokyo, Japan).

The PCR products after the second run were purified by using ISOSPIN PCR Product Kit (Nippon Gene, Tokyo, Japan) and sequenced by using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a model 3500 Genetic Analyzer (Applied Biosystems). The sequences were aligned and

Table 1. Primers and TaqMan probes for real-time PCR assays

	Name*	Sequence
Nakagawa *et al.* (current study)	FeLV_U3_exo_F	AACAGCAGAAGTTTCAAGGCC
	FeLV_U3_exo_R (2)	TTATAGCAA†AAAGCGCGG†G
	FeLV_U3_exo_probe	CCAGCAGTCTCCAGGCTCCCCA
Tandon *et al.* 2005	FeLV_U3_exo_F	AACAGCAGAAGTTTCAAGGCC
	FeLV_U3_exo_R	TTATAGCAG†AAAGCGCGC†G
	FeLV_U3_exo_probe	CCAGCAGTCTCCAGGCTCCCCA
Torres *et al.* 2005	FeLV_U3_re_F	AGTTCGACCTTCCGCCTCAT
	FeLV_U3_re_R	AGAAAGCGCGCGTACAGAAG
	FeLV_U3_re_probe	TAAACTAACCAATCCCCATGCCTCTCGC

*Top sequence, forward primer; middle, reverse primer; bottom, TaqMan probe.
†The underlined characters represent single-nucleotide polymorphisms at nucleotide positions 299 and 308, respectively.

compared by using GENETYX software (version 13; Genetyx, Tokyo, Japan). In addition, two FeLV U3 sequences (Glasgow-1 (M12500) and FeLV3281 (M18248)) that had been registered in GenBank were included in the analysis (Supplemental Fig. 1).

We performed *env*-based genotyping by sequencing as described previously[21] with modifications. Briefly, the entire *env* sequence was PCR-amplified from genomic DNA from the cats we sampled by using primers Fe-9S and Fe-7R, which are specific for *pol* and the 3´ U3 region, respectively. The PCR products were sequenced by using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and a model 3500 Genetic Analyzer (Applied Biosystems). Sequences were obtained from both strands of each PCR product for verification. The *env* sequence data were analyzed by using GENETYX version 13 software (Genetyx), and sequences were aligned by using Clustal W (http://clustalw.ddbj.nig.ac.jp/index.php?lang=en). A phylogenetic tree was constructed by using the maximum-likelihood method according to the Tamura–Nei model (MEGA7 software). In addition, the *env* sequences of FeLV strains, an endogenous FeLV, and a Friend murine leukemia virus strain that had been registered in GenBank were included in this analysis.

Real-time PCR analysis was performed by using StepOnePlus Real-Time PCR System (Applied Biosystems). Reaction conditions comprised initial denaturation at 95°C for 20 sec, followed by 40 amplification cycles consisting of denaturation at 95°C for 1 sec and annealing–extension at 60°C for 20 sec. Reaction mixtures consisted of 5 μL of template DNA (10 ng/μL), 10 μL of Premix Ex Taq (Takara Bio), 0.4 μL of each forward and reverse primer (10 μM) (Eurofins Genomics), 0.8 μl of 2.5 μM TaqMan probe (Life Technologies Japan, Tokyo, Japan), 0.4 μL of ROX Reference Dye (Takara Bio), and sufficient deionized water to bring the reaction volume to 20 μL. The sequences of the forward primers, reverse primers, and TaqMan probes are shown in Table 1.

We constructed a plasmid that contained part of the U3 region as a standard for real-time PCR analysis. The desired portion of U3 region was PCR-amplified by using the genomic DNA from a cat we sampled and the forward primer FeLV_U3_exo (2) and the reverse primer FeLV_U3_R (Table 1). PCR products were cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA); resulting plasmids were used to transform ECOS Competent *E. coli* DH5α cells (Nippon Gene). The plasmids were prepared by using GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich Japan, Tokyo, Japan). The sequence of the plasmid insert (that is, portion of U3 region) was

determined by using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and a model 3500 Genetic Analyzer (Applied Biosystems). The U3-containing plasmid was linearized with restriction enzymes *Spe*I and *Hind*III (New England Biolabs, Ipswich, MA) and then was purified by using ISOSPIN PCR Product Kit (Nippon Gene). The concentration of the linearized plasmid was determined by using a BioSpec-nano spectrophotometer (Shimazu, Kyoto, Japan). The copy number of the linearized plasmid was calculated according to its size.

Using nested PCR amplification, we identified 12 samples of genomic DNA from feline peripheral blood leukocytes that contained FeLV provirus. We PCR-amplified the full-length *env* sequences from these proviruses. Genotyping by sequencing and phylogenetic analysis revealed that 11 proviruses were classified as GI, while the other one was as GII out of 12 proviruses from the cat leukocytes. The provirus isolated from the FeLV3281 cells was classified as GIII (Fig. 1). The 12 FeLV *env* sequences that we identified from feline genomic DNA samples have been deposited in GenBank (Accession nos. LC332919, LC332920, LC332921, LC332922, LC332923, LC332924, LC332925, LC332926, LC332927, LC332928, LC332929 and LC332930).

We searched for SNPs in the U3 region that were linked to *env*-based genotypes because U3 is relatively conserved among FeLV strains. We compared the U3 sequences of 3 GI (IU119, IU128, and IU130) and the GII (IU144) proviruses we isolated with that from the GIII provirus in the FeLV3281 cell line. The analysis revealed multiple U3 SNPs. In particular, 2 SNPs, which were located at nucleotide positions 299 and 308 (referred to as SNP C299G and T308C, respectively; Supplemental Fig. 1), occur in the reverse primer of a previously developed real-time PCR assay used to quantify FeLV provirus[19].

We further investigated these SNPs *in silico* among proviruses for which *env*-based genotypes had been determined in previous studies[9,21] (Supplemental Table 1). This analysis of 74 GI and 11 GIII proviruses confirmed that SNPs C299G and T308C were conserved between these genotypes. Specifically, GI proviruses had C at nucleotide 299, whereas GIII proviruses had G; in addition, GI proviruses had T at nucleotide 308, whereas GIII proviruses had C at that position. However, the analysis of 5 GII proviruses revealed that 3 proviruses had G at nucleotide 299 and C at nucleotide 308, whereas the other 2 GII proviruses contained C and T at these positions, respectively.

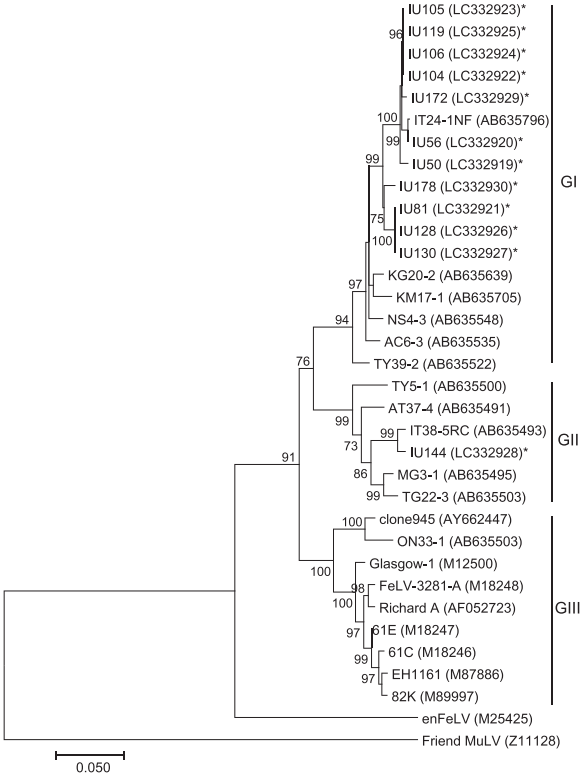

Fig. 1. Phylogenetic tree of *env* of feline leukemia virus (FeLV). The nucleotide sequences of *env* were analyzed by using the maximum-likelihood method (MEGA7 software). A bootstrap test was performed with 1000 replicates; bootstrap values $>70\%$ are indicated at branches. Reference sequences were obtained from GenBank; accession numbers are shown in parentheses. Asterisk indicates proviruses isolated in the current study. The *env* sequences were classified into 3 genotypes, as previously reported[21]. A Friend murine leukemia virus *env* was used as an out-group.

To rapidly differentiate GI FeLV proviruses from GIII proviruses, we developed a real-time PCR assay in which the reverse primer included the SNPs C299G and T308C (Table 1). We

Table 2. Specificity of real-time PCR assays against various *env*-based genotypes

Genotype	Sample	Threshold cycle*,†		
		Nakagawa *et al.* (current study)	Tandon *et al.* 2005	Torres *et al.* 2005
I	IU50	26.6	UD	34.1
	IU56	29.4	UD	UD
	IU81	21.5	UD	26.1
	IU104	20.5	UD	28.4
	IU105	19.2	UD	26.7
	IU106	21.1	UD	28.3
	IU119	23.1	UD	26.4
	IU128	25.0	UD	30.2
	IU130	21.9	UD	26.7
	IU172	18.5	UD	26.4
	IU178	24.3	UD	31.4
II	IU144	UD	20.4	24.3
III	FeLV3281	UD	17.6	19.8

*Mean of 2 replicate samples.
†UD, undetected: no amplification or threshold cycle $\geq$ 35.

confirmed the linearity and amplification efficiency of our real-time PCR assay by generating standard curves with 10-fold serial dilutions (10^1–10^7 copies) of the standard plasmid, which contained a portion of the U3 region of the LTR of FeLV (Supplemental Fig. 2). To assess the specificity of our real-time PCR assay, we applied it to the proviruses we isolated from feline peripheral blood leukocytes or FeLV3281 cells and compared the results with those from 2 previously developed real-time *env*-based PCR assays[19,20]. Our newly developed real-time PCR assay detected all GI proviruses but not those of the other genotypes (Table 2). In contrast, one previously developed real-time PCR[19] detected the GII and III proviruses but not any of the GI proviruses, whereas the other[20] detected all but one of the proviruses, regardless of genotype.

One limitation of the current study was only a few (one each) GII and GIII proviruses were used to assess the genotype specificity of the previous and current real-time PCR assays. This limitation arose because most FeLVs isolated in Japan belong to GI[21]. However, our *in silico* analysis of multiple strains isolated in Japan, Europe, and North and South America confirmed that the 2 SNPs we incorporated in the reverse primer were conserved between GI and GIII, thus suggesting that our new real-time PCR assay likely can discriminate between these *env*-based genotypes in the field.

It should be noted that our *in silico* analysis also suggested that our new real-time PCR assay would detect a subset of GII proviruses and could not discriminate between GI and GII. Since GII is a minor but non-negligible population isolated exclusively from Japan[21], further studies are needed to develop a real-time PCR assay to discriminate between GII and the other genotypes to fully investigate the relationship between *env*-based genotype and viral pathogenicity in FeLV infection.

Our current results showed that, due to sequence mismatch in the reverse primer, one of the previously developed real-time PCR assays failed to detect FeLV GI, which is predominant in Japan. This assay has been used to investigate whether provirus loads influence disease outcome in FeLV-infected cats[5]. To investigate such a relationship in Japan, we are improving our

SNP-based real-time PCR assay to quantify simultaneously both GI provirus and an internal reference gene.

In summary, we identified SNPs that are located in the long terminal repeat but linked to *env*-based genotypes, I and III. Our real-time PCR assay included a primer containing the SNP sites and detected GI proviruses but not GIII proviruses. Our new real-time PCR assay will enable rapid differentiation between FeLV genotypes, I and III. Such rapid genotyping will facilitate investigation of the relationship between FeLV genotype and viral pathogenicity in infected cats.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.67.1.103

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