NOTE *Virology*

Notch2 Transduction by Feline Leukemia Virus in a Naturally Infected Cat

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ABSTRACT. Feline leukemia virus (FeLV) induces neoplastic and nonneoplastic diseases in cats. The transduction of cellular genes by FeLV is sometimes observed and associated with neoplastic diseases including lymphoma and sarcoma. Here, we report the first natural case of feline *Notch2* transduction by FeLV in an infected cat with multicentric lymphoma and hypercalcemia. We cloned recombinant FeLVs harboring *Notch2* in the *env* gene. *Notch2* was able to activate expression of a reporter gene, similar to what was previously reported in cats with experimental FeLV-induced thymic lymphoma. Our findings suggest that the transduction of *Notch2* strongly correlates with FeLV-induced lymphoma.

KEY WORDS: feline leukemia virus, hypercalcemia, lymphoma, *Notch2*, transduction.

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Feline leukemia virus (FeLV), a gammaretrovirus that can cause a variety of both proliferative and degenerative diseases, is a major pathogen of feline lymphoma [4, 6]. The transduction and activation of cellular proto-oncogenes by FeLV are mechanisms associated with the occurrence of lymphomas and sarcomas. Some recombinant FeLVs harboring cellular sequences, such as the transcription factor *myc* [3, 5, 10, 16, 18–20, 26] and T-cell receptor β chain gene *tcr* [10], have been cloned from cats with naturally occurring lymphoma. FeLV, which transduces the intracellular region of *Notch2*, has been cloned from cats with experimental FeLV-induced thymic lymphoma [24].

Notch2 is a single-spanning transmembrane receptor that belongs to the Notch family of proteins, which play a role in cell differentiation and generation of tumors. The physical contact between cells expressing Notch ligands (e.g., delta-like ligands DLL1, 3 and 4 and Jagged1 and 2) and cells expressing the Notch protein induces proteolytic cleavage of Notch. This leads to release of the intracellular region of Notch into the nucleus, resulting in activation of responsive gene expression [reviewed in 11]. The active forms of Notch receptors have been reported in human patients with lymphoma and leukemia [8, 15, 27, 30]. Here, we report, for the first time, transduction of feline *Notch2* sequence by FeLV (*Notch2*-FeLV) in a naturally infected cat with multicentric lymphoma and hypercalcemia.

A 2-year-old, 2.0-kg, spayed female Japanese domestic cat was referred to the Veterinary Medical Center, The University of Tokyo, in 1995 with consecutive debilitation, dehydration and leanness. The cat was tested positive for FeLV p27-Gag antigen and diagnosed with multicentric lymphoma. Although the tumor had temporally gone into remission after chemotherapy, relapse occurred, and severe hypercalcemia was observed in its blood biochemistry profile (Table 1). Radiography showed extensive calcification in the pulmonary field and concurrent decalcification in the scapula and humerus. Despite treatment with furosemide, infusion of sodium chloride saline, porcine calcitonin (4 IU/kg) and salmon calcitonin (4 IU/kg) for hypercalcemia, little effective palliation was observed, and the cat died with neural manifestations on day 21. Marked invasion of the tumor cells was seen in the multiple tissues at necropsy.

We extracted DNA from the tumor tissue and amplified the entire *env* gene of the FeLV provirus using two PCRs as described previously and employing specific primer pairs (5′-CAT CGA GAT GGA AGG TCC AAC G-3′ (Fe-8S) and 5′-CAT GGT YGG TCY GGA TCG TAT TG-3′(Fe-3R) and 5′-GAG ACC TCT AGC GGC GGC CTA C-3′ (Fe-9S) and 5′-GTC AAC TGG GGA GCC TGG AGA C-3′ (Fe-7R)) [28]. Amplicons were cloned using Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA, U.S.A.). Two FeLV clones (KeyN2-1 and KeyN2-2) contained feline *Notch2*-like sequences; the nucleotide sequences of the clones were deposited in GenBank under accession numbers AB818695 and AB818696, respectively (Fig. 1). Both sequences contained

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Table 1. Blood tests for the cat with lymphoma

Complete blood count			Blood biochemistry profile		
	Patient	Reference range		Patient	Reference range
RBC (×10^6/μl)	6.59	5.00–10.00	BUN (mg/d*l*)	45.0	17.6–32.8
Ht (%)	28	24–45	Cre (mg/d*l*)	1.8	0.8–2.4
Hb (g/d*l*)	9.5	8.0–15.0	ALT (U/*l*)	199	12–130
TP (g/d*l*)	6.8	5.7–7.8	ALP (U/*l*)	1	14–111
PLT (×10^3/μl)	95	300–800	LDH (U/*l*)	711	0–798
WBC (×10^3/μl)	15.8	4.9–20.0	Ca (mg/d*l*)	17.3	8.8–11.9
Eos (%)	1	2–10	P (mmol/*l*)	8.0	2.6–6.0
Band (%)	0	0–2	Na (mmol/*l*)	148	147–156
Seg (%)	72	35–75	K (mmol/*l*)	4.2	3.4–4.6
Lym (%)	26	20–55	Cl (mmol/*l*)	115	107–120
Mono (%)	1	1–4			

ALP, alkaline phosphatase; ALT, alanine aminotransferase; Band, banded neutrophil; BUN, blood urea nitrogen; Ca, calcium concentration; Cl, chloride; Cre, creatinine; Eos, eosinophil; RBC, red blood cells; Ht, hematocrit; Hb, hemoglobin; K, potassium; LDH, lactate dehydrogenase; Lym, lymphocyte; Mono, monocyte; Na, sodium; P, phosphate; PLT, platelet; Seg, segmented neutrophil; TP, total protein; WBC, white blood cells.

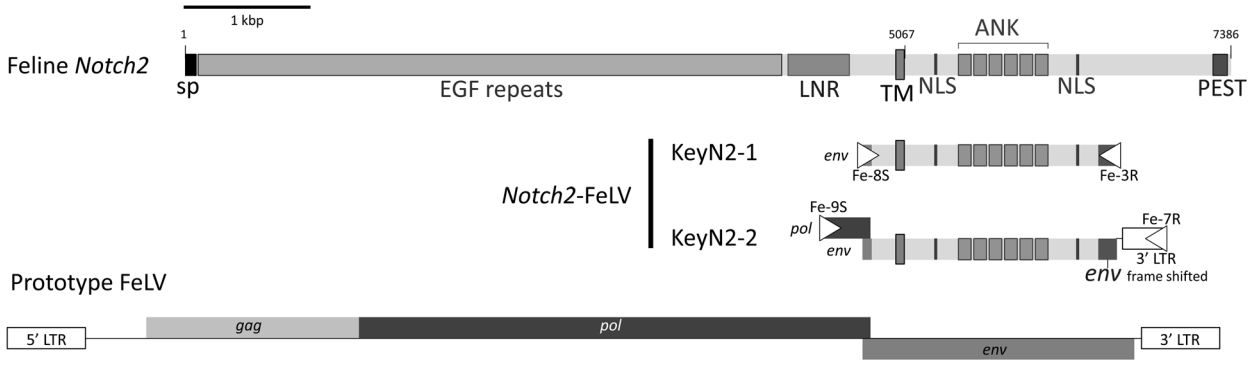

Fig. 1. Genetic structures of *Notch2*-FeLV. Schematic structures of the two clones of *Notch2*-FeLV (KeyN2-1 and KeyN2-2), feline *Notch2* and prototype FeLV provirus. Notch2 contains EGF (blue) and Lin-12-Notch repeats (LNR; pink) in its extracellular region and ANK repeats (orange), two NLSs (green) and proline/glutamic acid/serine/threonine-rich motifs (PEST; purple) in its intracellular region. TM; Notch2 transmembrane. Triangle indicates the primers used for cloning the two *Notch2*-FeLVs. sp, signal peptide.

the same recombinant junctions, along with 5′ and 3′ terminal sequences derived from FeLV *env* gene, and an intracellular region harboring transmembrane (TM) and ankyrin (ANK)-repeats of the feline *Notch2* gene. Both clones had short 23-amino-acid open reading frame (ORF), possibly derived from the FeLV *env* gene (Fig. 2A). A second ORF contained a sequence with a frame-shifted *env* sequence at its C-terminal, and this ORF possibly expresses viral Notch2 (v-Notch2) fusion protein (Fig. 2B). Other researchers have reported similar *Notch2* transduction during experimental infection of cats with FeLV 61E, a cloned virus, and have isolated four clones of *Notch2*-FeLV from two cats [24]. Three recombinants had the same 5′ junctions as those seen in our clones; however, the 3′ junctions were dissimilar. The second ORF of the recombinant v-Notch2 protein is translated by using the internal ribosome entry site (IRES) activity within the TM region of *Notch2* [14]. All variants isolated to date include the intracellular region of *Notch2* with functional ANK repeats and two nuclear location signals (NLSs). Such truncated expression of Notch receptors can lead to the constitutive activation of Notch signaling [reviewed in 12]. Direct repeat sequences of the enhancer or the upstream of the enhancer (URE) [21] were not seen in the LTR of KeyN2-2.

We further analyzed activation of Notch signaling pathway by the v-Notch2 protein using transient luciferase reporter assays. The predicted second ORF of clone KeyN2-2 was amplified by PCR using specific primers (5′-GAG GAT CCA TGG CGA AAC GAA AGC GTA A-3′ (Fe-250S) and 5′-TTG AAT TCT TAC AGG TCT TCT TCA GAG ATC AGT TTC TGT TCG CTG GAA GTC ATG GTT GG-3′ (Fe-223R), tagged with Myc at the C-terminus and then cloned into the pFUΔss expression vector [1]. The v-Notch2 protein was transiently expressed in HEK293T cells using Screen-Fect A (Wako, Osaka, Japan) as manufacturer's instructions (Fig. 3A). Co-transfection of our plasmid with pGa981-6

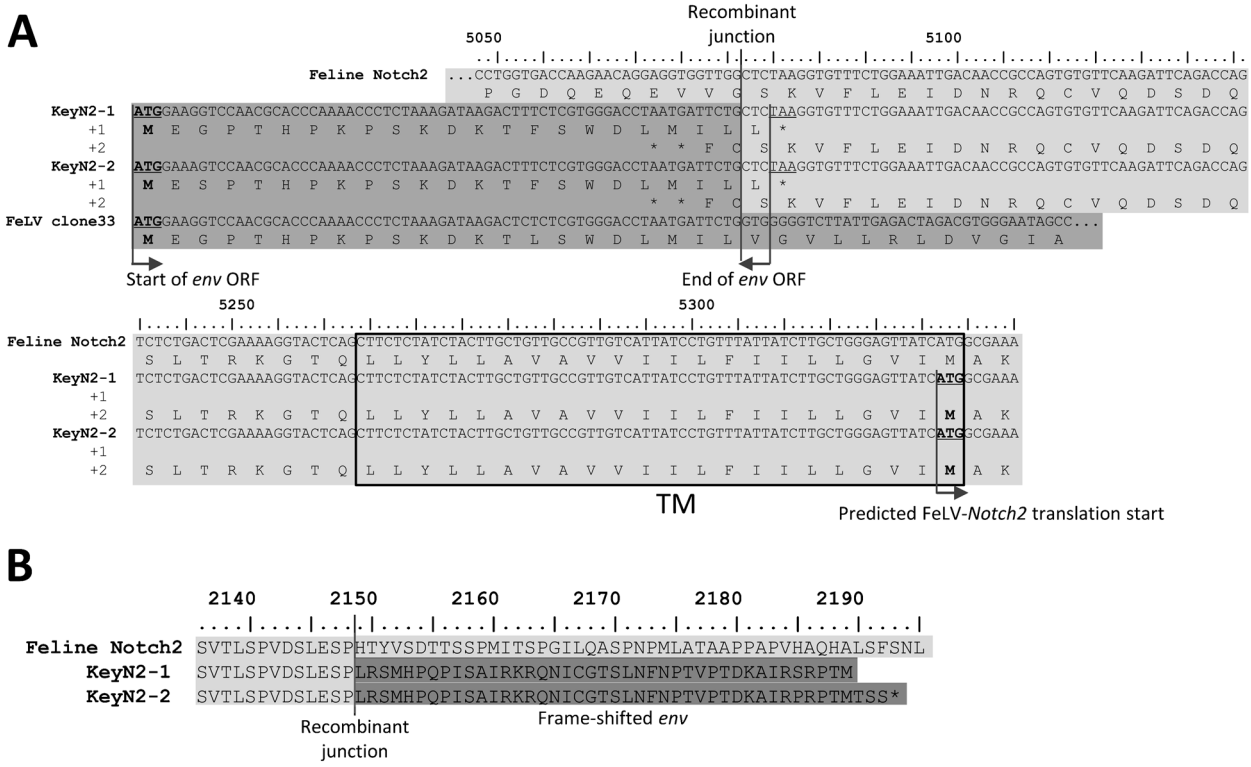

Fig. 2. The sequence alignment of *Notch2*-FeLV recombinant junctions flanking the 5′ (A) and 3′ (B) regions. Predicted start codons of the *env* gene and the recombinant *Notch2* are underlined and in bold. FeLV clone 33 (GenBank accession no. AB060732) [22] was used as a prototype FeLV reference sequence. The reading frame of the 3′ terminus of the *env* gene was frame-shifted (red). *Stop codon. TM; *Notch2* transmembrane.

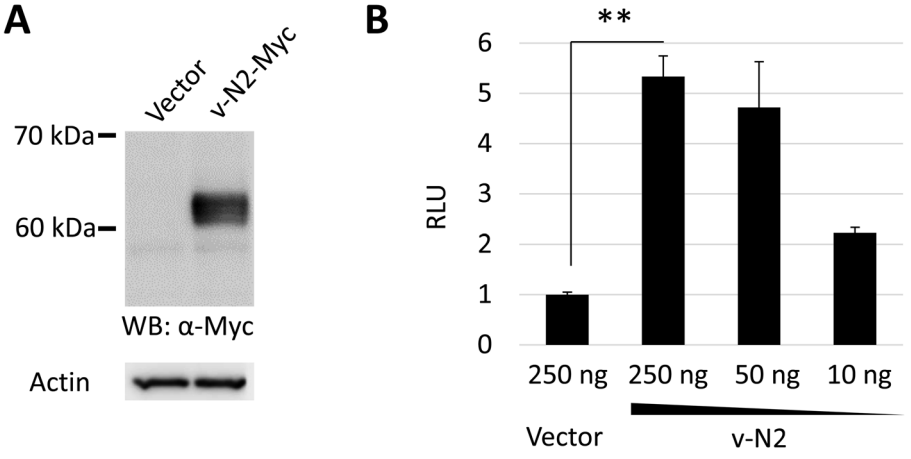

Fig. 3. Expression and activation of v-Notch2 protein. (A) Expression of Myc-tagged v-Notch2 proteins in transiently transfected HEK293T cells. HEK293T cells were transfected with pFUΔss expression vector (vector) [1] or pFUΔss-KeyN2-Myc (v-N2-Myc). Cells were collected after 48 hr, and total cell lysates were subjected to Western blotting analysis using mouse anti-Myc (Wako, Osaka, Japan) or mouse anti-β-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). (B) HEK293T cells in 24-well plate were co-transfected with pGa981-6 (50 *n*g), phRL-CMV (5 *n*g) and v-Notch2 expressing plasmids (v-N2). Luciferase assay was performed in triplicate, and the relative luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) at 48 hr post transfection. The relative luciferase unit (RLU) is shown relative to the negative control (vector). Error bars denote the standard deviation (SD). $^{**}P<0.01$ using unpaired *t*-test.

[13], a firefly luciferase reporter containing the RBP-Jκ binding promoter and phRL-CMV reference plasmid (Promega, Madison, WI, U.S.A.) that constitutively expresses renilla luciferase showed dose-dependent activation of the v-Notch2 protein (Fig. 3B).

Lymphoma and refractory hypercalcemia was observed in our cat. Hypercalcemia is commonly linked to malignancy in dogs and humans, especially in lymphoma [7, 23]. In most cases of feline lymphoma, elevation of the serum calcium concentration or parathyroid hormone-related peptide (PTHrP) is uncommon [2, 25]. Recently, an association between bone metabolism and Notch signaling has been revealed; RANKL-induced association of Notch2 and Jagged1 in pre-osteoclasts can lead to the differentiation into osteoclasts and activates osteoclastgenesis in them through the NF-κB pathway [9, 29]. Our v-Notch2 protein lacked extracellular region of Notch2. Therefore, v-Notch2 likely possesses the potential to activate osteoclastgenesis independent of RANKL stimulation when overexpressed in osteoclast/monocyte lineage cells.

Because of the recombination-prone property of gammaretroviruses, we observed numerous recombination events and various recombinant forms of FeLVs transducing cellular genes, including endogenous FeLV (enFeLV) [28] and ERV-DC [1]. The emergence of such recombinant viruses could alter the disease specificity, potential and outcome in FeLV-infected cats. Additionally, various numbers of proto-oncogenes, which function as a key regulator of proliferation and differentiation, have been historically identified in such recombinant gammaretroviruses. Although Notch2 has been recognized as tumor suppressor in some human tumors [17], transduction of *Notch2* gene seems to be a feasible mechanisms for FeLV-induced lymphomagenesis. Finally, our report may provide a new insight into the relationship between the Notch signaling pathway and humoral hypercalcemia.

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