**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.

**KEYWORDS:** 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 temporarily 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 neutral 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 TCC GGA TCG TAT TG-3' (Fe-8R)) [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
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].

Table 1. Blood tests for the cat with lymphoma

| Complete blood count | Blood biochemistry profile |
|----------------------|----------------------------|
| Patient | Reference range | Patient | Reference range |
| RBC (×10⁶/μl) | 6.59 | 5.00–10.00 | BUN (mg/dl) | 45.0 | 17.6–32.8 |
| Ht (%) | 28 | 24–45 | Cre (mg/dl) | 1.8 | 0.8–2.4 |
| Hb (g/dl) | 9.5 | 8.0–15.0 | ALT (U/l) | 199 | 12–130 |
| TP (g/dl) | 6.8 | 5.7–7.8 | ALP (U/l) | 1 | 14–111 |
| PLT (×10³/μl) | 95 | 300–800 | LDH (U/l) | 711 | 0–798 |
| WBC (+1⁰³/μl) | 15.8 | 4.9–20.0 | Ca (mg/dl) | 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.

Fig. 2. Genetic structures of the two recombinant clones of Notch2-FeLV (KeyN2-1 and KeyN2-2), along with the 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.
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 ng), phRL-CMV (5 ng) 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.
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