Clonality Analysis of Various Hematopoietic Disorders in Cats Naturally Infected with Feline Leukemia Virus

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Abstract. The clonality analysis of the bone marrow cells was carried out by detecting the integrated proviruses of feline leukemia virus (FeLV) to understand the pathogenesis of FeLV-associated hematopoietic disorders in cats. Bone marrow cells from 4 cases with acute myeloid leukemia (AML), 9 cases with myelodysplastic syndromes (MDS), 2 cases with pure red cell aplasia (PRCA) and 3 healthy carriers infected with FeLV were subjected to Southern blot analyses using an exogenous FeLV probe. Clonal hematoposisis was found in all the cases with AML and in 6 of the 9 cases with MDS, but not in the cases with both PRCA and healthy carriers infected with FeLV. In the 2 cases with MDS, it was thought that the same clones of the hematopoietic cells might proliferate before and after the progression of the disease irrespective of the changes of the hematological diagnoses by cytological examination. This study indicates that MDS in cats is a disease manifestation as a result of clonal proliferation of hematopoietic cells and can be recognized as a pre-leukemic state of AML.

Keywords: clonality, feline, feline leukemia virus, hematological disorder, myelodysplastic syndrome.


It is well recognized that erythrocytes, leukocytes and platelets originate from a single common hematopoietic stem cell such as a pluripotential stem cell in bone marrow. Since substantial numbers of hematopoietic stem cells are present in normal bone marrow, they constitute a polyclonal population in general. By contrast, because tumor cells generally are derived from a single cell that acquired malignant phenotype, monoclonal or oligoclonal cell proliferation is usually observed in neoplastic disorders. Therefore, analysis of the clonal composition of hematopoietic cell populations is important in the investigation of hematopoietic disorders [9, 11, 14, 20]. In human medicine, clonality analysis by inactivation/methylation analysis in female patients heterozygous for X-linked DNA polymorphism, such as the CAG repeat of the human androgen receptor (HUMARA) gene, can be used to explore the presence of clonally expanded cell population in various hematopoietic disorders including polycythemia vera, primary myelofibrosis, acute leukemia, myelodysplastic syndromes (MDS), and aplastic anemia (AA) [4, 11, 12].

In veterinary medicine, various hematopoietic disorders are frequently observed in cats infected with feline leukemia virus (FeLV) [10, 17, 21]. FeLV is a type-C retrovirus horizontally transmitted among domestic cat populations in natural conditions [17]. Persistent infection with FeLV is associated with induction of proliferative and degenerative diseases of various hematopoietic cell lineages in cats [25].

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The predominant form of the neoplastic disease associated with FeLV infection is thymic lymphoma, but a variety of acute myeloid leukemia (AML) and MDS are also frequently observed. It is well known that pure red cell aplasia (PRCA) and AA are also associated with FeLV infection [1, 2]. Since most of the field strains of FeLV do not contain oncogene transduced from the host genome, the tumorigenesis caused by FeLV infection is considered to be mediated through the interaction of FeLV and host cells [17, 21]. In the initial infection, integration of FeLV in target cells such as lymphoid and myeloid cells occurs at random sites in the host genome. On the other hand, in cats that have developed malignancies such as lymphoma and leukemia, a clonally expanded cell population can be shown by Southern blot analysis for T-cell receptor and immunoglobulin genes or integration of the FeLV proviral genome [13, 25]. Furthermore, in these tumors, clonal cell populations with respect to the proviral integration adjacent to oncogenes such as myc, bim-1, and myb have been observed [24, 25, 31, 32].

In small animal practice, one of the major problems in FeLV infection is nonregenerative anemia. Many of the cats with such nonregenerative anemia in FeLV infection can be diagnosed as PRCA, AA, MDS or AML [7, 18, 19, 21]. Among these disease, MDS is a controversial disease, characterized by peripheral blood cytopenia due to dysplastic changes in hematopoietic cells. Furthermore, a certain number of cats with MDS develop AML after the diagnosis of MDS [5, 28, 29]. Therefore, it has been required to investigate the pathogenesis of MDS, especially in its relation to AML. Based on these findings, it is conceivable that clonal-
ity analysis of hematopoietic cells should provide useful information on the pathogenesis of these hematological disorders especially on that of MDS. Because most cats with these diseases are infected with FeLV, the clonality analysis of hematopoietic cells can be performed by examination of the proviral integration of FeLV. The purpose of this study is to reveal the presence of clonal proliferation of hematopoietic cells in various feline hematopoietic disorders including neoplastic and non-neoplastic diseases by using the analysis of FeLV proviral integration in the bone marrow cells.

MATERIALS AND METHODS

Cases: Bone marrow cells were obtained from 18 cats infected with FeLV (Table 1). Three cats (Cases 1–3) were healthy asymptomatic carriers which had been experimentally inoculated with FeLV-A P17E2 strain [22, 24] 3 months before the sampling. These cats were clinically healthy and had no abnormal finding in their bone marrow and peripheral blood. The other 15 cats were patients referred to the Veterinary Medical Center, the University of Tokyo for diagnosis and treatment. The diagnoses were primarily based on detailed cytological examination of the bone marrow cells obtained by aspiration biopsy. By counting at least 300 bone marrow cells, the diagnoses of the disease were carried out according to the criteria of Jain et al. [19] essentially based on the French-American-British (FAB) classification for acute leukemia in humans [6]. The cases examined in this study included 2 cats with PRCA, 9 cats with MDS and 4 cats with AML. According to the human FAB subclassification for MDS and AML [6], the MDS cases were subclassified into refractory anemia (RA) (Cases 6–9), refractory anemia with excess of blasts (RAEB) (Cases 10 and 11), refractory anemia with excess of blasts in transformation (RAEB in T) (Case 12), and chronic myelomonocytic leukemia (CMML) (Cases 13 and 14), and the AML cases were subclassified into myeloblastic leukemia (M2) (Cases 15 and 16), monocytic leukemia (M5) (Case 17), and erythroleukemia (M6) (Case 18).

Two (Cases 9 and 14) of the 9 cases with MDS developed AML 1 week to 1 month after the diagnosis of MDS. In Case 9, bone marrow samples at the diagnosis of MDS and at subsequent AML (M0) were evaluated for the clonality of bone marrow cells. In Case 12 with RAEB in T, a bone marrow sample 3 months prior to the diagnosis of MDS was also obtained for clonality analysis. All of the 18 cats examined for clonality of bone marrow cells in this study were shown to be infected with FeLV. Infection with FeLV was identified by the presence of FeLV antigen in plasma samples by using an enzymed-linked immunosorbent assay-based commercial test kit (Petchek FeLV/FIV, IDEXX Laboratories, Portland, ME).

Probe: To detect FeLV proviral genomes in the chromosomal DNA, a probe specific to exogenous FeLV was prepared from a plasmid clone of FeLV-A, pJ7E2 [24]. The probe specific to exogenous FeLV corresponding to the U3 region of LTR was prepared by polymerase chain reaction (PCR). A commercially available PCR kit (Gene amp, Perkin-Elmer Cetus, Norwalk, CT) was used for PCR amplification. The primer sequences were 5'-TTAATCTAAGTA TGTTCCCATG-3' and 5'-CTGGGGAGCTGGAGACTGCT-3', complementary to the sequences upstream and downstream of the enhancer sequence of FeLV LTR, respectively [24]. Reaction mixtures contained 1x reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, and 0.001% gelatin), 200 mM dNTP, 2.5 units of AmpliTaq DNA polymerase, 1 µg of genomic DNA, and 0.5 µg of each primer. Amplification was achieved by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min. PCR products were directly cloned into a cloning vector (TA cloning kit, Invitrogen, San Diego, CA) and sequenced by the dyeodeoxy chain termination method (Auto Read Sequencing kit, Pharmacia, Uppsala, Sweden). The DNA fragment of 165 bp corresponding to the U3 region of the P17E2 clone was used as a probe specific to exogenous FeLV.

Southern blot hybridization: Bone marrow cells were obtained from the bone marrow aspiration biopsy or necropsy. High-molecular-weight cellular DNAs extracted from the bone marrow samples were analyzed by Southern blot hybridization. Samples of cellular DNAs (10 µg/lane) were digested with restriction endonuclease, EcoRI or BamHI, and subjected to 0.8% agarose gel electrophoresis. The DNAs were then transferred to nylon membrane filters and hybridized with the 32P-labeled exogenous FeLV-specific probe under relatively stringent conditions in a buffer containing 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5x Denhardt’s solution, and 1% sodium dodecyl sulfate at 65°C for 18 hr. The filters were washed 2 times in a solution containing 1x SSC and 0.1% SDS at 55°C and then exposed to X-ray films at –80°C for 3–4 days.

RESULTS

For detection of integrated FeLV provirus in the bone marrow cells, high-molecular-weight cellular DNA samples were digested with EcoRI which does not cut the proviral genome of general FeLV strains, or BamHI which has a single cutting site in the pol region of most FeLV strains. The digests were examined by Southern blot analysis with an FeLV probe corresponding to the LTR U3 region. In this analysis, a detectable band indicates the presence of a clonally expanded cell population with integrated FeLV provirus at a certain site in the host genome.

In asymptomatic healthy cats infected with FeLV (Cases 1–3), neither EcoRI nor BamHI digests gave any discrete band hybridized with the FeLV probe, but showed indistinct smears (Fig. 1 A, B). In the 2 cats with PRCA (Cases 4 and 5), there was no distinct band observed in the Southern hybridization analysis, a result similar to those in the asymptomatic carrier cats. These results indicated that there was no detectable clonally expanded cell population infected with FeLV in the bone marrow from asymptomatic carrier cats and cases with PRCA in this study.
**Fig. 1.** Southern blot analysis for the integration of proviral genome of FeLV in the bone marrow cells from cats with various hematopoietic disorders. Cellular DNAs extracted from the bone marrow cells were digested with *Eco*RI (A) or *Bam*HI (B) and subjected to Southern blot analysis with a probe specific to exogenous FeLV. Numbers of lanes are the case numbers. Cases 1–3, asymptomatic healthy carriers; Cases 4 and 5, PRCA; Cases 6–9, MDS with low blast counts (RA); Cases 10–14, MDS with high blast counts (RAEB, RAEB in T and CMML); Cases 15–18, AML. The DNA size marker of lambda DNA digested with *Hind*III is shown at the left of the panel.
Table 1. Clonality analysis of bone marrow cells in cats with various hematopoietic disorders

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Clonality status</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Healthy</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>Healthy</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>Healthy</td>
<td>−</td>
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<tr>
<td>4</td>
<td>PRCA</td>
<td>−</td>
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<tr>
<td>5</td>
<td>PRCA</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>MDS/RA</td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>MDS/RA</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>MDS/RA</td>
<td>+</td>
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<tr>
<td>9</td>
<td>MDS/RA</td>
<td>+</td>
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<tr>
<td>10</td>
<td>MDS/RAEB</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>MDS/RAEB</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>MDS/RAEB in T</td>
<td>+</td>
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<tr>
<td>13</td>
<td>MDS/CMMoL</td>
<td>−</td>
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<tr>
<td>14</td>
<td>MDS/CMMoL</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>AML/M2</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>AML/M2</td>
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<tr>
<td>17</td>
<td>AML/M5</td>
<td>+</td>
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<tr>
<td>18</td>
<td>AML/M6</td>
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+, detectable clonal hematopoiesis.
−, absence of detectable clonal hematopoiesis.

Of the 9 cats with MDS, 4 (Cases 6–9) were diagnosed as RA belonging to the low blast count group (blast cell counts less than 5% of the bone marrow cells and less than 1% in the peripheral blood nucleated cells), and 5 (Cases 10–14) were diagnosed as RAEB, RAEB in T, and CMMoL belonging to the high blast count group (blast cell counts more than 5% of the bone marrow cells and more than 1% of the peripheral blood nucleated cells). Of the 4 cases with RA, 2 cases (Cases 8 and 9) showed bands in the Southern blot analysis with the FeLV probe. The bone marrow DNA sample from Case 8 gave a 5.3-kb band in the EcoRI digest and 3 bands of 4.8–11.0 kb in the BamH1 digest. The sample from Case 9 showed a 3.2-kb band in the EcoRI digest and a 3.0-kb band in the BamH1 digest, but there was no detectable band hybridized with the FeLV probe in 2 other cases with RA (Cases 6 and 7). In the samples from 4 of the 5 cases with MDS of high blast count group (Cases 10, 11, 12, and 14), 3 to 6 discrete bands of 4.9–25.0 kb in the EcoRI digests and 5 to 10 bands of 2.0–12.5 kb in the BamH1 digests were seen in Southern blot analysis. In one case with CMMoL (Case 13), there was no distinct band hybridized with FeLV probe in both of the EcoRI and BamH1 digests. These results identified that there existed clonally expanded cell populations infected with FeLV in the bone marrow cells in 6 of the 9 cases with MDS.

Southern blot analysis of the samples from 4 cases of AML (Cases 15–18) revealed 2–4 bands of 5.7–15.0 kb in their EcoRI digests and 3–9 bands of 1.7–17.0 kb in their BamH1 digests.

Case 9 was diagnosed as RA at the first referral, but there remained questions about the diagnosis because the bone marrow was hypocellular and there were few cells with dysplastic changes in the bone marrow (Fig. 2a). This case rapidly progressed to AML/M0 1 month after the diagnosis of MDS (Fig. 2b). In Southern blot analysis with the FeLV probe, the bone marrow sample at the time of diagnosis of MDS/RA showed a band of 3.0 kb-BamH1 fragment, and that at the time of diagnosis of AML/M0 revealed 5 bands of 2.4–5.6 kb including a band of 3.0 kb in the BamH1 digest (Fig. 2c). These results indicated that the same clone existed at both stages diagnosed as MDS/RA and AML/M0 and the

Fig. 2. Progression from MDS/RA to AML/M0 in Case 9. Bone marrow aspiration cytology at the diagnosis of MDS/RA (a) and that at the diagnosis of AML/M0 (b). Wright-Giemsa staining, × 450. Southern blot analysis for FeLV integration with bone marrow DNA samples digested with BamH1 (c). Lane 1, DNA sample obtained at the diagnosis of MDS/RA; lane 2, DNA sample obtained at the diagnosis of AML/M0.
increased number of bands detected at the AML/M0 stage corresponded to the superinfection of the same clone with FeLV or clonal expansion of another bone marrow cells infected with FeLV.

At the time of the first admission, Case 12 was tentatively diagnosed as AA based on the hypocellular bone marrow and apparent lack of dysplastic change (Fig. 3 a). After 3 months, the diagnosis was changed to MDS/RAEB in T based on the cytology of the bone marrow cells showing an increased number of blast cells and dysplastic changes (Fig. 3 b). The DNA sample of this case at the diagnosis of suspected AA showed 7 BamHI bands of 2.7–12.0 kb in the Southern blot hybridization for FeLV integration. The band pattern at the diagnosis of MDS/RAEB in T was very similar to that at the diagnosis of AA (Fig. 3 c), indicating that the hematopoietic cell clone infected with FeLV shown at the stage of MDS/RAEB in T had already existed at the stage of suspected AA.

**DISCUSSION**

In the present study provirus integration of FeLV was investigated in the primary bone marrow cells from cats infected with FeLV. In our analysis, proviral integration of FeLV was random in the population of bone marrow cells derived from asymptomatic FeLV carrier cats and cats with PRCA, indicating the absence of detectable population of clonally expanded cells in the asymptomatic carrier state and PRCA. It was also indicated that PRCA in cats was a disease of a polyclonal cell population in an analysis for cellular mosaicism of glucose-6-phosphate dehydrogenase (G-6-PD) [3]. But because the method was only available in cats heterozygous for G-6-PD, reports which referred to the clonality of bone marrow cells in PRCA were limited to the cats experimentally infected with FeLV. In this study, it was indicated that the cats with naturally occurring PRCA had polyclonal hematopoiesis, as seen in the cats experimentally infected with FeLV.

Southern blot analysis in this study showed that leukemic cells derived from typical AML cases were clonally expanded cells in which the provirus genome was integrated into a certain site in the cat genome. Similar clonal hematopoiesis was proved in 6 of the 9 cats with MDS in the present study. These results showed that the dyshematopoiesis found in MDS had been caused by clonal proliferation of abnormal hematopoietic cells in the bone marrow, indicating the highly possible explanation that MDS is a preleukemic state of AML. Because even 2 of the 4 MDS cases with low blast counts had clonal hematopoiesis, therefore, it was thought that dyshematopoiesis in an early stage of MDS might be due to expansion of abnormal cell clones. In 3 of the 9 cats with MDS, however, clonal hematopoiesis was not detected by Southern blot analysis with FeLV probe, even though they had severe dyshematopoiesis. The reason why the clonal hematopoiesis was not detected in these MDS cases might be the polyclonal hematopoiesis as a mechanism for development of MDS or undetectable clonal hematopoiesis due to a decrease in the number of abnormal cell clones caused by a mechanism of apoptosis.

In human medicine it is recognized that increased apoptosis of hematopoietic cells is found in the bone marrow from patients with MDS [8, 15, 16, 27]. Furthermore, monoclonal hematopoiesis was seen in most MDS patients by X-inactivation/methylation analysis in female MDS patients heterozygous for X-linked DNA polymorphism of G-6-PD, phosphoglycerate kinase, hypoxanthine phosphoribosyltransferase and androgen receptor [4, 14, 20, 26]. Human MDS is therefore now considered to be a clonal hematopoietic disorder originating in a pluripotent stem cell having the ability to differentiate into erythrocytes, leukocytes and platelets [12, 14]. These findings indicate that MDS in cats
resembles that in humans with respect to the clonal origin of
the abnormal hematopoietic cells although a possible etio-
logic agent, FeLV, can be found only in cats.

Although it was difficult to confirm dysplastic changes in
the hypocellular bone marrow samples in cases 9 and 12 at
some stages of their disease, Southern hybridization analysis
of the bone marrow cells showed the presence of a clonal
hematopoietic cell population in this study. In veterinary
medicine, AA is generally recognized as a disorder with
depletion of hematopoietic cells in the bone marrow [21].
But Case 12 with suspected AA was found to progress to
MDS/RAEB in T, and the pattern of FeLV provirus integra-
tion at the AA stage was similar to that at the MDS/RAEB in
T stage. Therefore, it appears that some cases with AA may
have a features similar to those with MDS. In human medi-
cine, it was reported that some AA patients underwent MDS
or AML and that the bone marrow cells in AA showed a
molecular evidence of clonal hematopoiesis [23, 30, 34].
Further research is required to investigate the pathogenesis
of AA in relation to clonal hematological disorders including
MDS and AML.

In 7 cases with MDS or AML (Cases 8, 9, 10, 11, 12, 14
and 18), discrete bands shorter than 8.0 kb corresponding to
the length of common FeLV were detected in the Southern
blot analysis for FeLV with EcoRI digests. Because EcoRI
does not have a cutting site in most of the common FeLV
proviruses, these short bands may be due to some genetic
change in FeLV in these cats with MDS or AML. These
short bands might be derived from FeLV proviruses which
had some restriction site of EcoRI in its genome or those with
large deletion of the genome. Some deleted proviruses have
been reported in cats with AML [33]. FeLV-GM1/AB strain
which was isolated from a cat with AML had a large deletion
in its gag and pol genes [33].

Further effort is needed to characterized the proviral
genome of FeLV in the bone marrow from cats with MDS
and AML.

The present study disclosed the existence of clonal
hematopoiesis in FeLV-infected cats with hematopoietic dis-
orders such as MDS and AML. The clonal analysis
employed in this study will provide useful information not
only for the diagnosis of the hematopoietic disorders but also
for the further study on the pathogenesis of these diseases
associated with FeLV infection.

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