Molecular cytogenetic analysis of feline leukemia virus insertions in cat lymphoid tumor cells

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Abstract

This study was conducted to map the acquired proviral insertions in the chromosomal genome of feline lymphoid tumors induced by feline leukemia virus (FeLV). Chromosome specimens of the lymphoid tumor-derived cell lines and normal cat lymphocytes were subjected to fluorescence in situ hybridization and tyramide signal amplification, using an exogenous FeLV-A genome as a probe. Specific hybridization signals were detected only on the metaphase chromosomes of the tumor cells. Poisson's distribution-based statistics indicated that 6 chromosomal loci in each cell line showed FeLV integration. In the examination of metaphase chromosomes of FL-74, FT-1 and KO-1 cells, significant signals were detected on B2p15-p14, B2q11, D1p14, E1p14-p13, E1q12 and F2q16; A2p23-p22, B2p15-p14, B4p15-p14, D4q23-q24, E1p14-p13 and E2p13-p12; and A2p22, A2q22, B1p13, B1q13, D1p13 and D3p15-p14, respectively. Consistently, Southern blot hybridization using an FeLV LTR-U3 probe specific for exogenous FeLV revealed the presence of at least 6 copies of exogenous FeLV proviruses at different integration sites in each cell line. These results indicate that there may be common FeLV integration sites at least in A2p22 and B2p15-p14. The cytogenetic analysis used in this study can promptly screen FeLV insertions and provide tags for identifying the novel common integration site.

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1. Introduction

Feline leukemia virus (FeLV) is a type-C retrovirus associated with hematopoietic and lymphoid malignancies in cats. Acquired insertional mutagenesis is thought to be a major pathogenic mechanism underlying the malignancies induced by oncogenic retroviruses, including FeLV (Fujino et al., 2008). Identification of clonal proviral insertions of oncogenic retroviruses has led to the discovery of many tumor-associated genes (Jonkers and Berns, 1996; Li et al., 1999). Previous studies on FeLV-associated tumors have suggested that the integrated proviruses induce activation of cell growth-related genes leading to oncogenesis (Neil et al., 1984; Forrest et al., 1987; Miura et al., 1987, 1989; Levesque et al., 1990, 1991; Levy et al., 1993a,b; Tsujimoto et al., 1993; Tsatsanis et al., 1994; Fujino et al., 2009). Such insertional mutagenesis can contribute to the generation of a cell clone with growth advantage and an eventual malignant phenotype.

Thus far, only 6 common integration sites for FeLV proviruses have been identified in naturally occurring and experimentally induced feline lymphomas. Pioneering studies have reported insertional mutagenesis and over-expression of the c-myc proto-oncogene (Neil et al., 1984; Forrest et al., 1987; Miura et al., 1987, 1989; Levy et al., 1993b; Tsatsanis et al., 1994). Further, common FeLV-insertion loci were identified adjacent to proto-oncogenes, pim-1 (Tsatsanis et al., 1994) and fvi-2 encoding feline homolog of bmi-1 (Levy and Lobelle-Rich, 1992; Levy et al., 1993a,b; Tsatsanis et al., 1994). Several other unique common integration sites for FeLV have been identified as fvi-1 (Levesque et al., 1990, 1991), fit-1 (Tsujimoto et al., 1993) and frit-1 (Fujino et al., 2009). The frit-1 locus has been shown to be linked to the c-myc proto-oncogene (Tsujimoto et al., 1993; Tsatsanis et al., 1994; Barr et al., 1999). Further, the frit-1 locus is believed to be close to the ACVR1/ALK1, activin A receptor type II-like 1 gene, and proviral insertion at this locus may promote the expression of the gene (Fujino et al., 2009). Hence, acquired proviral insertions in FeLV-induced neoplasms can be used as tags to identify tumor-associated genes.

Advanced cytogenetic analysis employing fluorescence in situ hybridization (FISH) has made it possible to detect chromosomal proviral insertions of oncogenic retroviruses such as murine leukemia virus (MuLV) (Acar et al., 2000) and human T-cell leukemia/lymphoma virus type-I (Uemura et al., 1997; Ohshima et al., 1998). According to these studies, it is considered that cytogenetic detection of FeLV proviral insertions can lead to prompt determination of the map loci of proviruses in neoplastic cells with FeLV-induced neoplasms (Fujino et al., 2003). Because of extensive conserved synteny between human and cat chromosomes (Lyons et al., 1998), FISH hybridization can be applied for mapping proviral insertions in FeLV-induced neoplasms.
Table 1
Summary of chromosome aberrations and FeLV provirus integrations in 3 cell lines.

<table>
<thead>
<tr>
<th>Cell line (chromosome number, sex chromosome)</th>
<th>Chromosome aberration</th>
<th>Provirus insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL-74 (2n = 39, XY)</td>
<td>del<a href="p13-p12">B1</a>, del<a href="q24-q35">B1</a>, add<a href="p15">B2</a>, add<a href="p14">D1</a>, add<a href="p13">D2</a>, +D3, +D4, −F1, −F1, dup(F2)</td>
<td>B2p15-p14, B2q11, D1p14, E1p14-p13, E1q12, F2q16</td>
</tr>
<tr>
<td>KO-1 (2n = 41, XXX)</td>
<td>+B2, +F2</td>
<td>A2p22, A3q22, B1p13, B1q13, D1p13, D3p15-p14</td>
</tr>
</tbody>
</table>

al., 1997; O’Brien et al., 1999; Murphy et al., 2000), FeLV integration loci can be compared with the map loci in human chromosomes.

The present study was conducted to map the loci of somatically acquired FeLV proviral insertions in cells with FeLV-induced neoplasms by employing FISH analysis in order to investigate insertional mutagenesis.

2. Materials and methods

2.1. Cells

In this study, the following 3 feline lymphoid tumor cell lines were used; FL-74 (Theilen et al., 1969), FT-1 (Miura et al., 1987, 1989) and KO-1 (Fujino et al., 2004). FL-74 was derived from lymphoid leukemia and FT-1 and KO-1 were derived from thymic lymphomas occurring naturally in FeLV-infected cats. These cells were grown in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 0.01 mg/ml of colcemid (Demecolcin, Wako, Osaka, Japan) for 1 h, and then with 0.05 mg/ml of pepsin (Sigma) at 37 °C for 10 min. Hybridization was performed at 37 °C for 42 h in a moist chamber. After hybridization, the samples on the slides were first washed with a solution containing 50% formamide and 2× SSC, and then with that containing 1× SSC. Subsequently, tyramide signal amplification was performed as described previously (Acar et al., 2000) by using the TSA™ Biotin System (NEN™ Life Science Products, Boston, MA). These samples were incubated further with fluorescein isothiocyanate (FITC)-conjugated avidin (Roche Molecular Biochemicals) at 37 °C for 45 min. The incubated samples were rinsed with 2× SSC, and stained concurrently with antifade solutions (Intergen, Purchase, NY) with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to identify individual chromosomes. The stained samples were then observed under a fluorescence microscope. The chromosome loci were determined on the basis of the nomenclature given for feline Q-banded chromosomes (Cho et al., 1997c). Fluorescence signals of each locus were counted from at least 10 metaphase chromosomes and significance of the hybridization signals was analyzed statistically by Poisson’s distribution along with the 270-band stage feline karyotype (O’Brien and Nash, 1982). p-value for each locus was calculated from the numbers of signals, observed metaphase chromosomes and band stage (270) by Poisson’s law. A p-value of <0.05 was considered significant.

2.2. Chromosome preparations

Metaphase chromosomes were obtained from FL-74, FT-1 and KO-1 cell lines, and normal cat ConA-stimulated PBMCs. These cells maintained in RPMI-1640 medium containing 10% FBS were treated with 0.01 mg/ml of colcemid (Demecolcin, Wako, Osaka, Japan) for 2 h before harvest. The treated cells were resuspended in 0.075 M potassium chloride at 37 °C for 2 h before harvest. The treated cells were fixed in a 3:1 mixture of methanol and acetic acid on ice. After repeating the fixation process more than 3 times, the cell suspension was placed on glass slides and air-dried. To analyze the karyotype, chromosomes were stained with a combined solution of Hoechst 33258 and quinacrine mustard for Q-bandning essentially as described previously (Cho et al., 1997c).

2.3. Fluorescence in situ hybridization (FISH)

A plasmid clone of pFGA-2 containing an entire proviral genome of FeLV-A/Glasgow-1 (Stewart et al., 1986) was biotinylated by nick translation (Roche Molecular Biochemicals, Mannheim, Germany) for use as a probe. FISH was performed essentially as described previously (Fujino et al., 2001a,b, 2003).

In brief, the chromosome preparations were pretreated with 0.1 mg/ml of RNase A (Sigma) in 2× saline sodium citrate (SSC, 1× SSC contains 0.15 M sodium chloride and 15 mM sodium citrate) for 1 h, and then with 0.05 mg/ml of pepsin (Sigma) at 37 °C for 10 min. Target chromosomes were denatured in a solution containing 70% formamide and 2× SSC (pH 7.0) at 72 °C for 3 min, and immediately followed by ice-cold ethanol dehydration.

One milligram of the labeled DNA probe was mixed with 5 mg of salmon sperm DNA (Invitrogen, Carlsbad, CA) in 0.01 ml of the hybridization mixture, which was composed of 50% formamide, 10% dextran sulfate, 2× SSC and 0.01% bovine serum albumin (BSA), and then denatured at 75 °C for 10 min. After hybridization, the samples on the slides were first washed with a solution containing 50% formamide and 2× SSC, and then with that containing 1× SSC. Subsequently, tyramide signal amplification was performed as described previously (Acar et al., 2000) by using the TSA™ Biotin System (NEN™ Life Science Products, Boston, MA). These samples were incubated further with fluorescein isothiocyanate (FITC)-conjugated avidin (Roche Molecular Biochemicals) at 37 °C for 45 min. The incubated samples were rinsed with 2× SSC, and stained concurrently with antifade solutions (Intergen, Purchase, NY) with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to identify individual chromosomes. The stained samples were then observed under a fluorescence microscope. The chromosome loci were determined on the basis of the nomenclature given for feline Q-banded chromosomes (Cho et al., 1997c). Fluorescence signals of each locus were counted from at least 10 metaphase chromosomes and significance of the hybridization signals was analyzed statistically by Poisson’s distribution along with the 270-band stage feline karyotype (O’Brien and Nash, 1982). p-value for each locus was calculated from the numbers of signals, observed metaphase chromosomes and band stage (270) by Poisson’s law. A p-value of <0.05 was considered significant.

2.4. Southern blot hybridization

High-molecular-weight genomic DNA samples were extracted from FL-74, FT-1 and KO-1 cells, and the liver of a feline fetus. The samples of cultured cells and homogenized tissue were treated with a lysis buffer containing 0.02 mg/ml of proteinase K, 0.01 M Tris−hydrochloride (pH 8.0), 1 mM EDTA, 0.5% SDS and 0.01 mg/ml of RNase A at 37 °C for 24 h. Subsequently, DNAs were extracted with phenol and chloroform, and precipitated with ethanol. The DNAs (0.015 mg) were digested with 100 U of restriction enzymes, electrophoresed on 0.8% agarose gels, and then transferred onto nylon membranes. A probe specific for the exogenous FeLV genome was prepared from the long terminal repeat (LTR) U3 region of an exogenous FeLV clone, pJ7E2 (Miura et al., 1987). The DNA samples on the membrane were hybridized with the 32P-labeled probe in a hybridization solution containing 5× SSC, 1% SDS, 0.05 M Tris−hydrochloride (pH 7.6), 0.1 mg/ml of salmon sperm DNA, and 5× Denhardt’s solution (1× Denhardt’s solution contains 0.02% each of Ficoll type 400, polyvinyl pyrrolidone and BSA fraction V) at 62 °C for 16 h. After hybridization, the filters were washed 3 times with a solution containing 1× SSC and 0.1% SDS at 57 °C for 20 min and then subjected to autoradiography.

3. Results

3.1. Chromosome karyotypes

Twelve well-banded metaphase chromosomes of each cell line were analyzed in detail by IQ-banded cat karyotyping (Felis catus, 2n = 38). All the 3 cell lines showed chromosomal abnormalities
including numerical changes (Table 1). Structural abnormalities were found in the FL-74 and FT-1 cell lines. All the metaphases of the FL-74 cells had an abnormal chromosome number (2n = 39) with additional B2, D3 and D4 chromosomes, and loss of both F1 homologs (Fig. 1). Structural abnormalities were found in B1, B2, D1, D2 and F2 chromosomes. One B1 homolog showed a deletion at the distal short arm (p13-p12), and the other B1 homolog showed a deletion of an interstitial region of the long arm (q24-q35). Each homolog of B2, D1 and D2 chromosomes had unidentified fragments at its short-arm end. One F2 homolog showed duplication, which was possibly due to centromeric fusion. Therefore, the representative karyotype of FL-74 cell line was designated as [39, XY, del(B1)(p13-p12), del(B1)(q24-q35), add(B2)(p15), add(D1)(p14), add(D2)(p13), add(D3), add(D4), -F1, -F1, dup(F2)].

All the metaphases of the FT-1 cells showed an abnormal chromosome number (2n = 39) with an additional B2 chromosome and marker chromosome and a monosomy of sex chromosomes (Fig. 2). An F1 homolog showed a structural abnormality of duplication, which occurred because of binding with the apex each other. Therefore, the representative karyotype of the FT-1 cell line was designated as [39, X, +B2, dup(F1), +mar].

All the metaphases of the KO-1 cells showed hyperdiploidy (2n = 41) because of the trisomies of B2, F2 and X chromosomes (Fig. 3). In contrast to the 2 earlier described cell lines, structural abnormality was not observed in this cell line. Therefore, the
representative karyotype of the KO-1 cell line was designated as [41, XXX, +B2, +F2]. Arrows indicate chromosomal aberrations of extra copies of B2, F2 and X.

### 3.2. Detection of FeLV proviruses in chromosomes by FISH

Significant hybridization delineating FeLV provirus integration sites was detected in the metaphase preparations from the FL-74, FT-1 and KO-1 cells (summarized in Table 1), but not in those from normal cat PBMCs. Chromosomal regions positive for FeLV integration could be identified in these cell lines on the basis of Poisson's distribution (p-value in parentheses indicated the probability that the signal was not significant).

In the examination of 25 metaphase chromosomes of the FL-74 cells, significant signals were detected on B2p15-p14 (p < 10⁻⁴), B2q11 (p < 10⁻⁸), D1p14 (p < 10⁻⁵), E1p14-p13 (p < 10⁻⁴), E1q12 (p < 10⁻⁴) and F2q16 (p < 10⁻⁵) (Fig. 4). The signals on D1p14 were detected at the abnormal region corresponding to the junction of the apex of the short arm and an additional unidentified fragment. The signals on F2q16 were detected on both the authentic and duplicate chromosomes.

In the examination of 10 metaphase chromosomes of the KO-1 cells, significant signals were detected on A2p23-p22 (p < 10⁻⁸), B2p15-p14 (p < 10⁻²⁸), B4p15-p14 (p < 10⁻¹⁷), D4q23-q24 (p < 10⁻³⁸), E1p14-p13 (p < 10⁻²³) and E2p13-p12 (p < 10⁻²⁸) (Fig. 5).

In the examination of 160 metaphase chromosomes of the FT-1 cells, significant signals were detected on A2p23-p22 (p < 10⁻²⁸), B2p15-p14 (p < 10⁻²²), E1p14-p13 (p < 10⁻²³) and E2p13-p12 (p < 10⁻²⁸) (Fig. 5).

### 3.3. Southern blot analysis for exogenous FeLV proviral genome

The copy number of integrated exogenous FeLV proviral genome was examined by Southern blot hybridization using a probe specific for exogenous FeLV (Fig. 7). The FL-74, FT-1 and KO-1 DNAs digested with BamHI, which has a cutting site in the pol gene of most exogenous FeLV isolates (Mullins et al., 1980; Casey et al., 1981; Stewart et al., 1986), gave 12 discrete bands corresponding to the DNA fragments containing the 5′ or 3′ LTR U3 region and adjunctive cellular flanking DNA sequences. Normal cat liver DNA did not give any detectable band. The Southern blot analysis results showed that these 3 cell lines comprised clonal cell populations with exogenous FeLV proviral integrations and revealed the copy numbers of the proviruses. The BamHI digest of FL-74 DNA gave 12 discrete bands of 18, 9.4, 8.0, 5.9, 4.8, 4.4, 4.2, 3.6, 3.4, 2.4, 2.2 and 1.9 kb. The FT-1 DNA gave 12 discrete bands of 15, 9.2, 7.1, 6.2, 4.9, 4.6, 4.0, 3.5, 3.0, 2.6, 2.3 and 2.1 kb. The KO-1 cell DNA gave 12 discrete bands of 6.6, 6.0, 5.0, 4.1, 2.7, 2.3, 2.1, 1.7, 1.6, 1.5, 1.4 and 1.3 kb. The Southern blot analysis results of the BamHI-digested DNA revealed the presence of at least 6 copies of exogenous FeLV proviral genomes at different integration sites in each of the 3 cell lines, since each band conceivably corresponded to a DNA fragment containing a 5′ or 3′ LTR region plus their flanking sequences. These results regarding the number of integrated FeLV proviruses could be consistent with those of the FISH analysis.

### 4. Discussion

The present FISH analysis appeared to generate positive signals only for exogenous FeLV proviral sequences since no significant hybridization signal was observed on normal cat chromosomes. In addition, the number of significant hybridization signals observed in the FISH analysis could be almost equal to the copy number of exogenous FeLV proviral sequences detected by Southern blot analysis in the 3 cell lines. Although the feline genome contains endogenous FeLV-like elements, the FeLV-A proviral sequence is less homologous to these elements as compared to most FeLV-B proviral sequences containing recombinant endogenous FeLV env (Roy-Burman, 1995). Use of FeLV-B isolates as probes might enable detection of both endogenous and exogenous FeLV insertions. The use of FeLV-A provirus probe in the analysis could only enable detection of exogenous FeLV insertions. There have been several reports on the detection of integrated oncogenic retroviruses by FISH analysis (Uemura et al., 1997; Ohshima et al., 1998; Acar et al., 2000). Detection of integrated FeLV provirus by FISH analysis in this study was considered to be useful for identifying the FeLV-integrated loci on cat chromosomes.
Exogenous FeLV proviral sequences were found in at least 6 chromosomes in each cell line examined in this study. On these chromosomes, some tumor-associated genes and common retroviral integration sites have been mapped (O’Brien et al., 1993, 1997a,b, 1999; Murphy et al., 2000). On the short arm of chromosome A2 where FeLV insertions have been observed in FT-1 and KO-1 cell lines, RAF1 has been mapped (O’Brien et al., 1993). On chromosome B1 where FeLV insertions have been detected on two loci in KO-1 cell line, KIT and IL2 have been mapped (O’Brien et al., 1993; Murphy et al., 2000). On B2 chromosome where FeLV insertions have been detected in FL-74 and FT-1 cell lines (two loci in the FL-74 cell line), FIT1, MOS, CDKN1A, PIM1, ROS1, MYB and FYN have been mapped (O’Brien et al., 1997a,b; Murphy et al., 2000). On chromosome D1 where FeLV insertions have been detected in FL-74 and
KO-1 cell lines, *HRAS*, *FGF3* and *ETS1* have been mapped (O’Brien et al., 1993, 1997a,b). On chromosome E1 where FeLV insertions have been observed in FL-74 and FT-1 cell lines (two loci in the FL-74 cell line), TP53 and *HIC1* have been mapped (Cho et al., 1997a; O’Brien et al., 1997a,b; Murphy et al., 2000). Since TP53 has been mapped regionally on E1p14-p13 (Cho et al., 1997a) which is one of the loci of integrated FeLV provirus in the FL-74 and FT-1 cells, it can be indicated that the FeLV provirus may have disrupted the tumor suppressor gene. Integrated FeLV proviruses may activate known or unknown tumor-associated genes adjacent to the integration sites since LTR of FeLV has a potential to induce transcriptional activation of certain cellular genes (Ghosh and Faller, 1999; Ghosh et al., 2000). The mapped positions of A2p22 and B2p15-p14 that are the overlapping FeLV insertion loci among the cell lines might contain the common FeLV integration sites.

In a previous study, FT-1 cells were shown to have an FeLV insertion upstream of c-myc (Miura et al., 1989). However, in this study FeLV integration was not found on the c-myc locus, F2q21.2 (Cho et al., 1997b). Such a contradictory result may be derived from a certain chromosomal translocation, failure of the detection by FISH analysis, or change in cell population during long-term cultivation. Similarly, slight discrepancy was observed between the present and previous results (Miura et al., 1989) of Southern blot hybridization performed using the probe specific for exogenous FeLV. The genomic status of FT-1 and FL-74 cells may be altered since they produce consistently infectious FeLV particles.
Meanwhile, the genomic status of KO-1 cells can remain undisrupted by cultivation since the cells are non-producers of FeLV particles and the results of Southern blot hybridization analysis and chromosome karyotyping performed in the present study are similar to those of the original study (Fujino et al., 2004). Hence, it can be recommended to apply the analysis to clinical cases of neoplasms or FeLV non-producer tumor cell lines for understanding FeLV-related spontaneous alterations.

In the present study, chromosome karyotypings showed some abnormalities in all the 3 cell lines. Hyperdiploidy of chromosome B2 was observed among all the 3 cell lines. Since several tumor-associated genes have been mapped on chromosome B2 as described above, aberrations of this chromosome in the 3 cell lines might be related to tumor development. In a previous cytogenetic study, FL-74 cell line had monosomies of A2, E2 and F2, trisomy of E1, loss of E3 and F1 chromosome pairs, an additional X chromo-
some, and several marker chromosomes (Gulino, 1992). However, such aberrations in the FL–74 cell line were not observed in this study. Possible explanations of this inconsistency are that the cell population changed during long-term cultivation, or the improved quality of karyotype analysis of Q-banded cat chromosomes (Cho et al., 1997c) made it possible to identify the chromosomal abnormalities. Interestingly, a hybridization signal of possible FeLV provirus insertion was observed at the locus of D1p14 in the FL–74 cells; this locus was the junction of the apex of the short arm and an additional unidentified fragment. The aberration of D1 chromosome in the FL–74 cells might be related to FeLV provirus insertion.

A previous study reported a large-scale analysis of proviral insertion sites in MuLV-induced leukemia cells by long-template inverse polymerase chain reaction to clone and sequence a large numbers of proviral insertion sites (Li et al., 1995). Although this method can be applicable to the analysis of FeLV insertions, it will be difficult to identify the sequences adjacent to the proviral insertion sites until the whole cat genome is successfully sequenced. FeLV-induced spontaneous malignancies have been frequently observed in veterinary medicine and only 6 genomic common integration sites associated with oncogenesis have been identified for FeLV. The FISH technique employed in this study will provide valuable molecular tags to reveal unidentified oncogenes and/or tumor-suppressor genes through the identification of common integration loci in multiple FeLV-associated tumor cells as well as in clinical cases.

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