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Identification of a novel common proviral integration site, *flit*-1, in feline leukemia virus induced thymic lymphoma

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ABSTRACT

A new proviral integration site for feline leukemia virus (FeLV), termed *flit*-1, was identified from feline thymic lymphoma. Among 35 FeLV-related tumors examined, 5 of 25 thymic lymphomas demonstrated proviral insertion within *flit*-1 locus whereas none of four alimentary and five multicentric lymphomas and one T-lymphoid leukemia examined had rearrangement in this region. Extensive sequence analysis has shown that *flit*-1, which is noncoding, is conserved on human chromosome 12 and mouse chromosome 15. The human and murine homologs of *flit*-1 are positioned approximately 30-kb upstream to activin-A receptor type II-like 1 (*ACVRL1/ALK1*) gene. Expression of *ACVRL1* mRNA was examined in two of five lymphomas with *flit*-1 rearrangement and detected in both of the two whereas normal thymuses and seven lymphoid tumors without *flit*-1 rearrangement had no detectable expression. Therefore, *flit*-1 appears to represent a novel FeLV proviral common integration domain that may influence lymphomagenesis as insertional mutagenesis.

Introduction

Retroviruses are associated with a variety of malignancies and provide powerful model systems for probing the biological and molecular mechanisms of tumorigenesis. For nonacute transforming type-C retroviruses such as feline leukemia virus (FeLV), the induction of tumors involves long latency period and multiple cooperative changes in growth control mechanisms (Fujino et al., 2008). Proviral insertional mutagenesis is well accepted as one of the most important steps in the process of retroviral tumorigenesis. Insertions near oncogenes occur by chance as a consequence of the normal viral life cycle (Mikkers and Berns, 2003). However, when a provirus integrates near a gene controlling growth and alters its expression, the host cell may have a selective growth advantage. Expansion of this cell eventually leads to the formation of a clonal tumor. Pioneering studies of avian leukosis virus (ALV)-induced bursal lymphomas revealed that c-*myc* proto-oncogene is activated by proviral integration and therefore initiated extensive studies of this oncogene and many others (Hayward et al., 1981). Studies with rodents have also identified more than a hundred integration sites by murine leukemia virus (MuLV) (Mikkers and Berns, 2003).

Compared to MuLV studies, only a few FeLV integration sites have been identified (Fujino et al., 2008). FeLV is a replication-competent type-C retrovirus that is associated with a variety of neoplastic diseases in domestic cats, of which T-cell lymphoma is the most common form (Fujino et al., 2003, 2004; Linenberger and Abkowitz, 1995; Neil et al., 1991). Like other mammalian leukemia retroviruses, common subgroups of FeLV carry no transforming gene in its genome with the exception of minor variants containing *myc* (Fujino et al., 2008; Linenberger and Abkowitz, 1995). One mechanism of FeLV tumorigenesis is believed to be due to the interactions between viral and host genetic information, such as insertional mutagenesis (Fujino et al., 2003, 2008; Hisasue et al., 2000, 2001; Linenberger and Abkowitz, 1995; Neil et al., 1991; Roy-Burman, 1995).

Early studies have gathered evidence indicating that c-*myc* proto-oncogene was activated by either viral transduction or proviral insertional mutagenesis (Forrest et al., 1987; Levy et al., 1993b, 1984; Miura et al., 1987, 1989; Neil et al., 1984; Tsatsanis et al., 1994). By studying the genetic loci interrupted by proviruses in tumors induced by FeLV-*myc* viruses, two other common integration sites were identified. One of these was *flvi*-2, which was interrupted by FeLV in about 24% T-cell lymphomas (Levy and Lobelle-Rich, 1992; Levy et al., 1993b; Tsatsanis et al., 1994). It was later proved to be a feline orthologue of *bmi*-1, which encoded a gene for a nuclear protein of the

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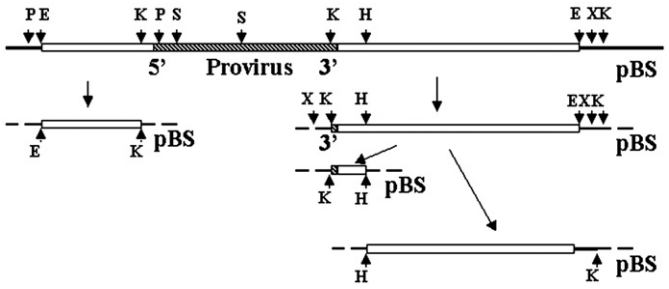

Fig. 1. Restriction endonuclease map of clone A5 and strategy of subcloning into pBS vector. The empty boxes indicate the cellular regions flanking the cloned proviral sequence, which is shown in dashed box. The solid lines represent the first pBS vector, and the broken lines indicate the vectors used for subcloning. The orientation of the provirus is as shown. The 5′ cellular fragment was cloned into *Eco*RI–*Kpn*I sites of pBS. The 3′ cellular region was first cloned into pBS using the KpnI sites. Subsequently, the 3′ cellular fragment was double digested into two pieces with KpnI and HindIII and cloned into pBS separately. Symbols: E, *Eco*RI; K, *Kpn*I; H, *Hind*III; P, *Pst*I; X, *Xho*I; S, *Sst*I.

zinc finger family. It has been speculated that *bmi*-1 gene product may act as a *myc* collaborator in the induction of B- and T-cell lymphomas (Haupt et al., 1991; Levy et al., 1993a; van Lohuizen et al., 1991). Another site, *fit*-1, was only shown to be interrupted in 8% of tumors (Tsatsanis et al., 1994). Although its coding potential has not been characterized, the provirus inserted in this site has been demonstrated to exert a long-range effect on feline orthologue of c-*myb* proto-oncogene (Barr et al., 1999; Hanlon et al., 2003; Tsujimoto et al., 1993). Besides these two loci, *pim*-1, which is a common integration site by MuLV and encodes an oncogenic serine/threonine kinase, was also shown to be frequently interrupted in FeLV-induced tumors (Tsatsanis et al., 1994; Wang et al., 2001). Additionally, an uncharacterized locus, *flvi*-1, has been shown to be interrupted by FeLV insertion in some feline lymphomas of the spleen (Levesque et al., 1990, 1991). Overall, much fewer FeLV common integration sites have been identified in lymphoid tumors as compared to MuLV studies. Therefore, the identification of important common integration sites and corresponding cellular proto-oncogenes in FeLV-induced lymphomas and the exploration of the functional roles of their gene products remain very important in unraveling the mechanisms of FeLV tumorigenesis. These studies, in turn, may lead to the discovery of orthologous genes important in tumorigenesis in human and thus contribute to a better understanding of retroviral tumorigenesis in human. In this regard, we initiated the present study and characterized a new common integration site by FeLV in thymic lymphomas.

Results

Identification of a new FeLV proviral integration site in a thymic lymphoma

The insertional mutagenesis hypothesis implicates that slow retroviruses, which do not carry oncogenes in their genomes, induce tumors by integrations into particular loci in host DNA thereby increasing or interrupting expression of cellular genes. Consistent findings of proviral sequences around the same locus in different tumors suggest this locus may encode a gene that is important in the multistep process of tumorigenesis (Mikkers and Berns, 2003). To search for such loci, cloning of proviral integration sites from one thymic tumor was attempted. A phagemid DNA library was established by linking λ DASH II vector to the *Eco*RI digested genomic DNA fragments that were prepared from a thymic tumor of cat 4746-5. *Eco*RI restriction enzyme was used to obtain the proviral sequences along with the cellular flanking regions because there was no *Eco*RI restriction site in all known FeLV proviral clones. The resulting phage DNA library was then screened with the exU3 probe that is specific for all known exogenous FeLV LTR sequences (Fujino et al., 2003, 2004; Hisasue et al., 2000; Miura et al., 1989).

With this strategy, two integration sites were identified. These two fragments in λ DASH II were subsequently cloned into the EcoRI site of pBS vector. Sequence analysis revealed that c-*myc* gene resided in one of the cloned integration sites. Another clone was named as A5 and the restriction enzyme site map was shown in Fig. 1. To facilitate identification of the cloned integration site carried by clone A5, its 5′ cellular region was ligated to *Eco*RI–*Kpn*I site of pBS, and the 3′ cellular region to the KpnI–KpnI site. The clone containing 3′ cellular region was further cut into two pieces and cloned into *Kpn*I–*Hind*III and *Hind*III–*Kpn*I sites of pBS (Fig. 1). Sequences within different fragments were determined first by primers on the pBS vector. Then various primers were designed based on these sequences for progressive DNA sequencing.

The 5′ LTR of the integrated provirus was sequenced and the comparison of its sequence with that of F6A, an FeLV subgroup A provirus (Donahue et al., 1988), revealed that A5 harbored an exogenous FeLV containing a 73-bp direct repeat around LTR core region (data not shown). Sequencing *env* gene of the provirus confirmed that A5 carried the FeLV subgroup A provirus. These two lines of evidence indicated that the cloned provirus was exogenous in origin and was kept in the cat genome during the tumor formation.

Clone A5 carried about 6.5-kb cellular sequence with 2-kb flanking the 5′ provirus and 4.5-kb downstream of the 3′ provirus. Both 5′ and 3′ cellular regions (based on the orientation of the provirus) were sequenced. Extensive search using the NCBI Genebank BLAST database revealed no significant homology to any known proto-oncogenes or tumor suppressor genes.

Examination of the integration locus in other FeLV-induced tumors

If the cloned integration site is involved in the induction of tumors by FeLV, then it is likely that this particular locus could also be interrupted by proviruses in other independent FeLV-related tumors. To test this possibility, naturally occurring FeLV-related lymphomas in twenty independent cats, experimentally induced lymphomas in eleven independent cats in addition to the lymphoma from cat 4746-5 and three T-lymphoid tumor cell lines were recruited (Table 1). Among those, 23 were thymic lymphomas, 4 were alimentary lymphomas, and 5 were multicentric lymphomas. T-cell tumor cell lines included FL-74 derived from FeLV-related T-lymphoid leukemia (Theilen et al., 1969), FT-1 derived from FeLV-related thymic lymphoma (Miura et al., 1987) and KO-1 derived from FeLV-related thymic lymphoma (Fujino et al., 2004). High molecular weight DNA was isolated from these tumor tissues and cells and digested with either SstI or EcoRI. SstI cuts twice in FeLV genome at the positions 634 and 3738 according to the F6A sequence, and EcoRI does not cut FeLV provirus at all. DNA fragments were separated in 0.8% agarose gels and

Table 1
FeLV-positive feline lymphomas examined in the present study

Source	Number examined	Number with *flit*-1 insertion
Thymic lymphoma (T-cell)		
Natural	11	2 (18%)
Experimental (FeLV-A/Rickard)	12	3 (25%)[a]
Cell line (FT-1, KO-1)	2	0
Multicentric lymphoma		
Natural	5	0
Alimentary lymphoma		
Natural	4	0
T-lymphoid leukemia		
Cell line (FL-74)	1	0

[a] One of these also exhibits c-*myc* insertion.

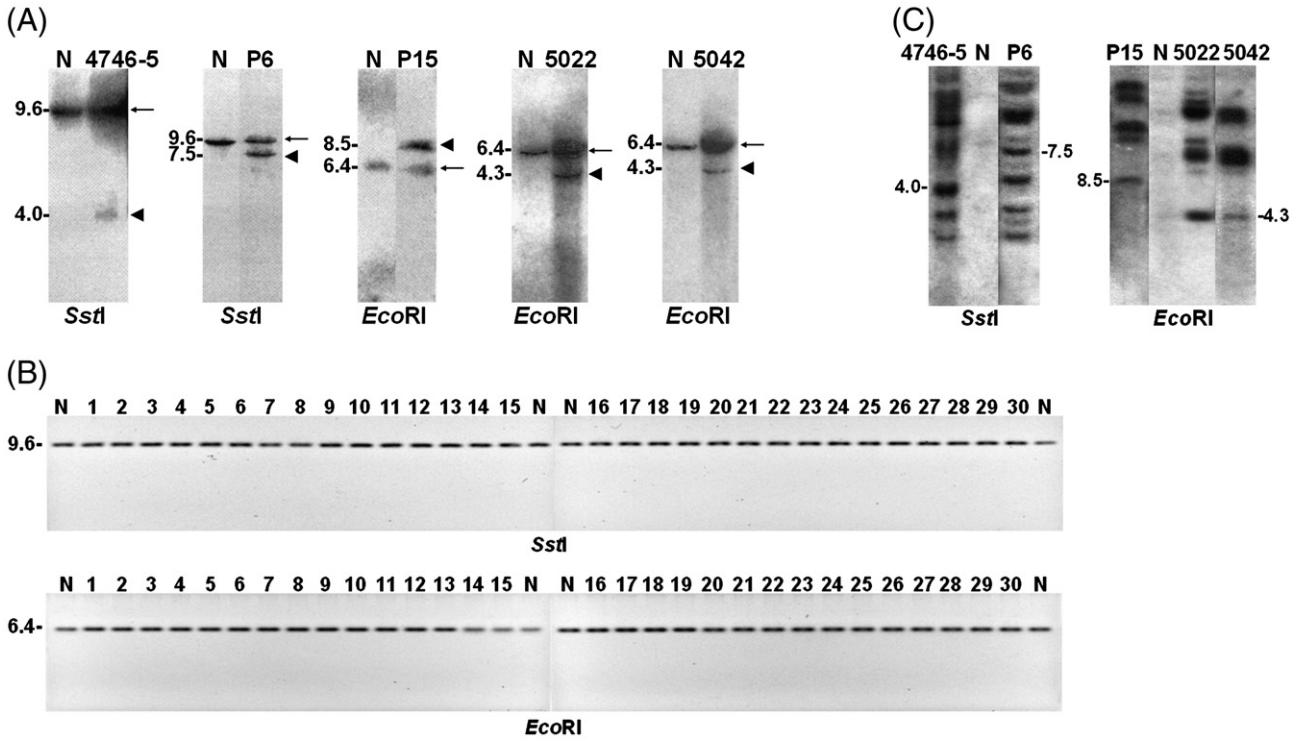

Fig. 2. Southern blot analyses for *flit*-1 insertion. High molecular weight DNAs were isolated and digested with either SstI or EcoRI. The resulting filters were then hybridized to a probe representing the 5′ EcoRI–KpnI cellular region of clone A5 (A and B) or the LTR-U3 specific for exogenous FeLV (C). Five thymic lymphoma samples carried *flit*-1 rearrangement (A) due to the integration of FeLV proviruses (C). None of other lymphoma samples were observed with *flit*-1 rearrangement (B). FeLV-positive twelve experimentally induced thymic lymphomas (4746-5, 5022 and 5042 on A and C, and 1 to 9 on B), eleven naturally occurring thymic lymphomas (P6 and P15 on A and C, and 10 to 18 on B), five naturally occurring multicentric lymphomas (19 to 23 on B), four naturally occurring alimentary lymphomas (24 to 27 on B) and three T-lymphoid tumor cell lines (28 of FL-74, 29 of FT-1 and 30 of KO-1 on B) were subjected. N stands for genomic DNA extracted from normal SPF cat fetus liver tissues as germline and FeLV-negative controls. The approximate molecular weight of individual bands is marked at the side. Arrows denote the uninterrupted cellular fragments, and arrowheads indicate the rearranged regions due to the proviral integration (A).

hybridized to a probe representing the sequence of 5′ cellular EcoRI–KpnI region of clone A5. As shown in Figs. 2A and B, the probe recognized normal *Sst*I fragments of approximately 9.6-kb in SPF fetus and all tumor tissues. In the 4746-5 and P6 tumor DNA samples, additional 4.0-kb and 7.5-kb fragments were identified, respectively (Fig. 2A). Similarly, while the same probe recognized normal cellular *Eco*RI fragments of approximately 6.4-kb in the normal and tumor tissues (Figs. 2A and B), altered fragments of 8.5-kb, 4.3-kb and 4.3-kb were also identified in the P15, 5022 and 5042 tumor DNAs, respectively (Fig. 2A). Therefore, including the original tumor where the A5 was cloned, five independent thymic lymphomas showed rearrangement around this locus (Table 1). Meanwhile, the membranes that had all the tumor DNA samples were stripped and rehybridized with the exU3 probe. This exogenous-specific proviral LTR-U3 probe also identified the additional bands aside from the normal cellular fragments (Fig. 2C). The additional 4.3-kb bands in 5022 and 5042 tumor DNAs were shown to be included proviral or cellular genomes with some nucleotide change or deletion because standard exogenous FeLV isolates had no EcoRI site and thus they are usually detected as bands longer than 8.5-kb. These results indicated that the integration site carried by clone A5 is a common proviral integration site in FeLV-induced tumors. This locus has been named as *flit*-1, an acronym for feline leukemia virus integration site in thymic lymphoma-1.

To exclude the possibility that *flit*-1 may carry known common FeLV proviral integration sites in the flanking region, *flit*-1 was compared with c-*myc*, *pim*-1, *flvi*-2, *flvi*-1 and *fit*-1. Although approximately 10-kb of both 5′ and 3′ flanking sequences of *flit*-1 (GenBank/EMBL/DDBJ accession number AB378061 and AB378062) were isolated by the adaptor ligation-based PCR-mediated genome walking strategy, they disagreed with feline sequences of c-*myc* (Stewart et al., 1986) and *pim*-1 (GenBank/EMBL/DDBJ accession number AB073748). Moreover, the membranes that had the tumor DNA samples carrying *flit*-1 insertion were stripped and rehybridized with c-*myc* and *pim*-1 probes (Fig. 3). The results showed that four of the five tumors carried the germline configuration of c-*myc* and *pim*-1 and the size of them was different from that of *flit*-1. The one tumor from cat 4746-5 had the rearrangement of c-*myc* and germline configuration of *pim*-1. Meanwhile, several XhoI-digested SPF feline fetus and tumor DNAs were hybridized to the *flvi*-2/*bmi*-1 DNA probe (Levy and Lobelle-Rich, 1992; Levy et al., 1993a,b) (Fig. 3). A 12-kb germline fragment instead of the 11-kb *flit*-1 band was detected. In

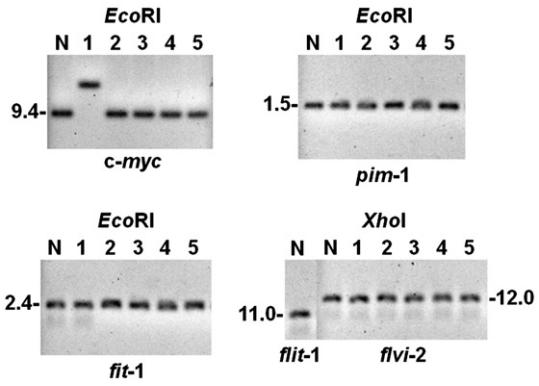

Fig. 3. Southern blot analyses for common integration sites of FeLV. High molecular weight DNAs were isolated and digested with either EcoRI or XhoI. The resulting filters were then hybridized to a probe of c-*myc*, *pim*-1, *fit*-1, *flit*-1 or *flvi*-2. Five thymic lymphomas carrying *flit*-1 insertion (1, 4746-5; 2, P6; 3, P15; 4, 5022; 5, 5042) and a normal SPF cat fetus liver (N) as a germline control were subjected. One thymic lymphoma sample (1, 4746-5) carried c-*myc* rearrangement. The approximate molecular weight of individual bands is marked at the side.

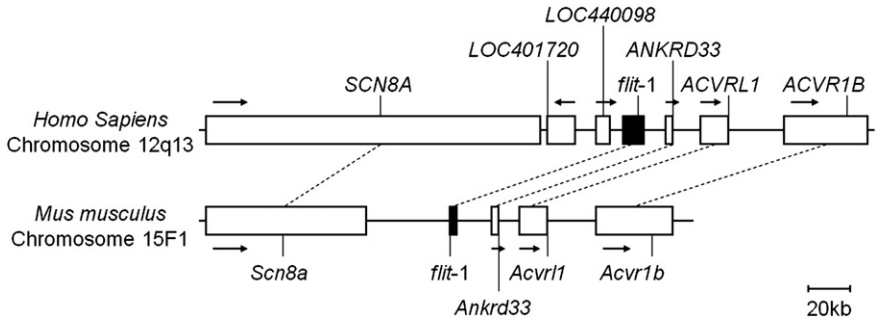

Fig. 4. Comparative genomic structure of the human chromosome 12q13 and mouse chromosome 15F1 surrounding *flit*-1 domain. The FeLV proviral integration sites, *flit*-1 (closed boxes), which have been located by BLAST searching of the draft human and mouse genome sequences are also depicted. The open boxes indicate annotated genes from the public domain of human and murine sequences. Horizontal arrows represent the orientation of the genes. Diagonal lines drawn between the human and murine orthologues indicate genes of homology.

addition, it has been reported that EcoRI fragments of *flvi*-1 and *fit*-1 are about 5.4-kb and 2.4-kb (Fig. 3), respectively (Levesque et al., 1990; Tsujimoto et al., 1993), whereas *flit*-1 EcoRI fragment is about 6.4-kb. Therefore, *flit*-1 is believed to be a novel integration site identified in FeLV-induced thymic lymphomas.

Evolutionary conservation and chromosomal assignment of flit-1

The FeLV insertion of *flit*-1 locus in 5 independent thymic lymphomas suggested that interruption of *flit*-1 site may lead to alteration of the expression of important cellular genes that are critical in the control of cell growth and differentiation. Due to the lack of complete feline genome sequences, Genome BLAST search using NCBI Genebank human and murine database was performed. Both 5′ and 3′ flanking cellular regions including extended sequences of clone A5 were used as queries in the search. In the search using human genome, one and three matches were yielded from 5′ and 3′ cellular query sequences, respectively. All matches assigned *flit*-1 locus to human chromosome 12q13 (Fig. 4). The matching sequences were shown to have 77 to 87% homology to corresponding human chromosome sequences. In the search using murine genome, three matches were yielded from 3′ cellular query sequences whereas there was no match from the 5′ cellular sequence. The matches could be assigned to mouse chromosome 15F1 (Fig. 4), which showed 83 to 88% homology to the corresponding sequences. Since feline chromosome B4 is partly conserved the synteny relationship with human chromosome 12 and mouse chromosome 15 (O'Brien et al., 1999), feline ortholog of *flit*-1 is supposed to be mapped on feline chromosome B4 although chromosomal assignment is needed. The human and murine homologs of *flit*-1 have been positioned approximately 20-kb upstream to *ANKRD33* which is a hypothetical gene and predicted to encode a transcription factor containing ankyrin repeat motifs. Since it has been shown that provirus can exert long-range effect to the genes located more than 100-kb away from the original integration site (Hanlon et al., 2003), we also searched genes located 100-kb upstream or downstream of *flit*-1 locus. *SCN8A* which encodes a member of the voltage-gated sodium channel (Burgess et al., 1995), was found to be located upstream of both human and murine *flit*-1 loci. Downstream of *flit*-1 site, it was identified with two activin-A receptor genes, *ACVRL1* (*ALK1*) and *ACVR1B* (*ALK4*), encoding cell-surface receptors for the transforming growth factor (TGF)-beta of ligands (Attisano et al., 1993; ten Dijke et al., 1993), as well.

Altered cellular gene expression by FeLV insertion in flit-1

Since identical genes are located near the *flit*-1 site on human and murine chromosomes, there could be homologous genes around *flit*-1 on feline chromosomal DNA. One of the genes, *ACVRL1* (GenBank/EMBL/DDBJ accession number AB378063), was shown to be important in the process of cell growth and differentiation. To investigate the mRNA expression of the gene in normal and neoplastic lymphoid cells, total RNA samples from a normal placenta of one SPF cat as a positive control, thymuses of two SPF cats, two normal T-lymphoid cell lines, three FeLV-induced T-lymphoid tumor cell lines and six independent FeLV-induced thymic lymphoma tissues were subjected to RT-PCR analysis. The mRNA expression of *ACVRL1* was distinctly detected in a placenta and lymphoma tissues of cat 5042 and 5022 that have FeLV provirus insertion in *flit*-1 whereas it was undetectable in thymuses, all cell lines and other lymphoma tissues that have no FeLV provirus insertion in *flit*-1 (Fig. 5). The results obtained here indicated that the expression of the cellular gene nearby *flit*-1, e.g. *ACVRL1*, could be positively influenced by the FeLV provirus insertion in *flit*-1.

Discussion

One mechanism of slow retroviral tumorigenesis is proposed as insertional mutagenesis where a provirus inserts near a cellular gene that is important for cell differentiation or proliferation and leads to aberrant cellular gene expression (Mikkers and Berns, 2003). As a representative of this virus family, FeLV has been found to target certain genetic loci in lymphomas of T-cell origin, such as c-*myc*, *flvi*-1, *flvi*-2, *pim*-1, and *fit*-1 (Fujino et al., 2008; Levesque et al., 1990; Levy

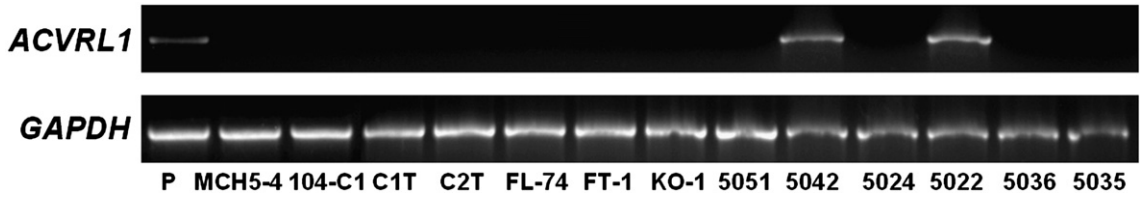

Fig. 5. Expression of *ACVRL1* mRNA in various feline cells and tissues. Total RNA samples isolated from a normal placenta of a SPF cat, two feline normal T-cell lines (MCH5-4 and 104C1), thymuses of two SPF cats (C1T and C2T), three FeLV-related T-lymphoid tumor cell lines (FL-74, FT-1 and KO-1), and six independent FeLV-induced thymic lymphomas (5051, 5042, 5024, 5022, 5036 and 5035) were subjected to RT-PCR analysis for *ACVRL1* mRNA. The mRNA expression of *ACVRL1* was distinctly detected in a placenta as a positive control and lymphomas of 5042 and 5022 that have FeLV provirus insertion in *flit*-1 whereas it was undetectable in the others that have no FeLV provirus insertion in *flit*-1. RT-PCR for *GAPDH* mRNA was used as an internal control.

and Lobelle-Rich, 1992; Miura et al., 1987; Tsatsanis et al., 1994; Tsujimoto et al., 1993). The study here reported the identification of a novel common integration site by FeLV, named *flit*-1, in thymic lymphomas.

The *flit*-1 locus was isolated from the experimentally induced thymic lymphoma of cat 4746-5 that had been challenged by FeLV Rickard strain subgroup A (Pandey et al., 1995; Sheets et al., 1992). The provirus that resided within this locus was confirmed to be exogenous FeLV-A and contained an enhancer duplication of 73-bp around the LTR-U3 core region. This tandem duplication could result from mutation during the process of viral replication. There are several studies that the tandem direct repeat around the core enhancer of the FeLV LTR has been frequently observed in thymic lymphomas and conferred an enhancer function upon gene expression (Chandhasin et al., 2004; Miura et al., 1989; Nishigaki et al., 1997; Plumb et al., 1991), suggesting its association with tumorigenic potential in lymphoid cells.

Approximately 10-kb of both 5′ and 3′ cellular flanking regions of *flit*-1 has been sequenced, respectively, and extensive Genebank database search did not reveal significant nucleotide homology of *flit*-1 with any of the known genes related to tumorigenesis including c-*myc* and *pim*-1, also reported as FeLV common integration sites. Hybridization and comparative analyses for previously identified FeLV common integration sites of *flvi*-1, *flvi*-2 and *fit*-1 were also shown to light up different germline fragments from *flit*-1. Therefore, *flit*-1 is a new FeLV integration site identified in the present study.

Total 35 FeLV-induced lymphoid neoplasms were examined and carried no *flit*-1 insertion of the FeLV provirus with the exception of some thymic lymphomas. Interestingly, including the original tumor from where the *flit*-1 locus was isolated, the FeLV integration was observed in 5 of 23 thymic lymphoma tissues (the frequency was 21.7%) and 25 thymic lymphomas including two cell lines (it was 20.0%) examined. Among these, the tumors from cat 4746-5, 5022 and 5042 were induced by challenging an FeLV-A Rickard strain preparation and only one from cat 4746-5 harbored proviral insertion near c-*myc* gene. The tumors of P6 and P15 were naturally occurring thymic lymphomas and contained no FeLV integration around c-*myc*. These results indicate that (1) proviral integration into *flit*-1 locus can be associated with generation of thymic lymphoma in cats; (2) unlike *flvi*-2 or *fit*-1 (Barr et al., 1999; Hanlon et al., 2003; Levy et al., 1993a; Tsujimoto et al., 1993), gene product of *flit*-1 insertion may not have a direct collaborating function with c-*myc*.

Identification of *flit*-1 as a frequently targeted domain of FeLV integration in thymic lymphoma suggests that *flit*-1 provides a function in target cells which confers a selective growth advantage or otherwise contributes to induce malignant transformation. In this respect, it is likely that *flit*-1 is located near a gene that is critical in the control of cell growth and differentiation and the insertion may influence expression of the gene. To identify key candidate gene(s) involved in FeLV-related lymphomagenesis, extended flanking regions of *flit*-1 were sequenced and Genome Blast search was performed using human and mouse genome database. It was demonstrated that *flit*-1 could be significantly assigned to approximately 30-kb and 80-kb upstream of *ACVRL1* (*ALK1*) and *ACVR1B* (*ALK4*), respectively, and 50-kb downstream of *SCN8A* on both human chromosome 12q13 and mouse chromosome 15F1. The ligand of both *ACVRL1* and *ACVR1B* is activin-A, which belongs to TGF-β superfamily and is a multi-functional cytokine that regulates cell growth and differentiation (Attisano et al., 1993; ten Dijke et al., 1993). The mutation of *ACVRL1* has been closely related with hereditary hemorrhagic telangiectasia (Johnson et al., 1996; Urness et al., 2000). The analysis of mRNA expression indicated that upregulated expression of *ACVRL1* by FeLV insertion into *flit*-1 might contribute to obtain growth advantage of the cell and thus be one of steps of tumorigenesis. This result can be a pioneer that *ACVRL1* may have potential to be associated with tumorigenesis.

The present study demonstrated that *flit*-1 locus was a frequently targeted genetic site in FeLV-induced thymic lymphomas and the insertion might have an effect on expression of the nearby gene, such as *ACVRL1*. The significance to malignant induction of insertional mutagenesis of these regions may be related to the action of particular retroviruses or to the induction of particular tumor types. In further studies, it becomes important to extend the functional study of nearby genes in lymphoid tumor induction to other species, such as humans.

Materials and methods

Subgenomic cloning

Subgenomic library screening was performed as described previously (Fujino et al., 2001a,b). Genomic DNA was purified from the thymic tumor tissue of cat 4746-5, which was challenged by an FeLV-A Rickard strain preparation (Pandey et al., 1995; Sheets et al., 1992). Since EcoRI does not cut into FeLV proviruses, a subgenomic library was constructed by ligation of 8- to 20-kb EcoRI fragments to the λ DASH II phagemid vector (Stratagene, La Jolla, CA). The exU3, which is specific for the U3 region of all known exogenous FeLV long terminal repeat (LTR) sequences (Fujino et al., 2003, 2004; Hisasue et al., 2000; Miura et al., 1989), was used as a probe to screen the phage library. Proviral clones containing cat genomic sequences were identified and subcloned into pBluescript (pBS) vector (Stratagene). One of the clones, A5, was determined later in this study to harbor a frequently targeted genetic locus by FeLV integration by Southern blot analysis. For further sequence analysis, a 5′ cellular sequence of A5 was subcloned into EcoRI–KpnI site of pBS vector, and a 3′ cellular region was first cloned into the KpnI–KpnI site of pBS vector and then subsequently cloned into KpnI–HindIII and HindIII–KpnI sites of pBS vectors, respectively. Sequences within individual fragment subcloned from the A5 plasmid were determined first by primers T3 and T7 on the pBS vector. Multiple primers were subsequently designed based on these sequences for further sequencing analysis.

Southern blot analysis

High molecular weight genomic DNA samples were extracted from naturally occurring or experimentally induced thymic, alimentary, or multicentric lymphomas of 32 independent FeLV infected cats, FL-74 (Theilen et al., 1969), FT-1 (Miura et al., 1987) and KO-1 (Fujino et al., 2004) cells, and several specific pathogen free (SPF) feline fetus liver tissues. These specimens were treated with a lysis buffer containing 0.02 mg of proteinase K per ml, 0.01 M Tris–hydrochloride (pH 8.0), 1 mM ehthylenediamine tetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS) and 0.01 mg of RNase A per ml at 37 °C for 24 h. Then, the DNA samples were extracted with phenol and chloroform, and precipitated with ethanol. The DNA samples (15 μg/lane) were digested with 100 U of restriction enzymes EcoRI or SstI, electrophoresed through 0.8% agarose gels, and transferred onto nylon membranes. The DNA samples on the membranes were hybridized with a ^{32}P-labelled A5-5 probe, which was the 5′ EcoRI–KpnI cellular fragment of A5 clone, in a hybridization solution containing 5× SSC, 1% SDS, 0.05 M Tris–hydrochloride (pH 7.6), 0.1 mg of salmon sperm DNA (Sigma, St. Louis, MO) per ml and 5× Denhardt's solution (1× Denhardt's solution consisted of each 0.02% of Ficoll type 400, polyvinyl pyrrolidine and bovine serum albumin fraction V) at 62–65 °C for 16 h. After hybridization, the filters were washed 3 times with a solution containing 1× SSC and 0.1% SDS at 57–60 °C for 30 min and subjected to autoradiography. The blots that contain the DNA samples with its rearrangement were stripped and hybridized to the exU3 probe. Some of the membranes were also stripped and hybridized with c-*myc*, *fit*-1, *flvi*-2 and *pim*-1 probes (Levy and Lobelle-Rich, 1992; Levy et al., 1993a; Tsatsanis et al., 1994; Tsujimoto et al., 1993), respectively. The feline *pim*-1 cDNA has been cloned

previously (GenBank/EMBL/DDBJ accession number AB073748), and its probe was generated based on the feline sequence.

Adaptor ligation-based PCR-mediated genome walking

The isolation of the unknown 5′ and 3′ cellular flanking sequences of clone A5 were achieved using an adaptor ligation-based PCR-mediated genome walking strategy. This splinkerette allows the amplification between a primer annealing in a known part of the sequence and a nearby restriction site laying out of the known part of the sequence (Devon et al., 1995). The protocol has been modified from its original; in that way that the restriction enzyme BamHI, EcoRI, HindIII, PstI and XbaI were used to create an overhang for ligation of the corresponding splinker. Annealing of splinkers was achieved by heating proper amounts of splnktop (5′ CGA ATC GTA ACC GTT CGT ACG AGA ATT CGT ACG AGA ATC GCT GTC CTC TCC AAC GAG CCA AGA 3′) with splnk BamHI (5′ GAT CTC TTG GCT CGT TTT TTT TTG CAA AAA 3′), splnk EcoRI (5′ AAT TTC TTG GCT CGT TTT TTT TTG CAA AAA 3′), splnk HindIII (5′ AGC TTC TTG GCT CGT TTT TTT TTG CAA AAA 3′), splnk PstI (5′ ACG TTC TTG GCT CGT TTT TTT TTG CAA AAA 3′) or splnk XbaI (5′ CTA GTC TTG GCT CGT TTT TTT TTG CAA AAA 3′) (20 μM each) in 20 μl of Tris, pH 7.4 and 5 mM $MgCl_2$ at 90 °C and cooling on the bench top for 20 min. A volume of 5 μg genome DNA was digested in a 20 μl reaction volume with 10 U of a same restriction enzyme as the adaptor for 16 h at 37 °C. The reaction was terminated by heat inactivation for 15 min at 90 °C. Four microliters of the digested DNA (1 μg) and 6 μl of the annealed splinker were mixed with 1 U of T4 DNA ligase in a final volume of 20 μl, incubated overnight at 15 °C and then, terminated for 20 min at 80 °C. A nested PCR was performed for amplification of the desired fragments. The primer splk0 (5′ CGAATCGTAACCGTTCGTACGAGAA 3′) was used in the first PCR, which annealed to the complement of the longer strand that was generated in the first PCR cycle by priming of a gene-specific primer. The primer splk1 (5′ TCGTACGAGAATCGCTGTCCTCTCC 3′) was used in the second PCR, together with the nested gene-specific primer. Each of the gene-specific primers was subsequently designed based on obtained flanking sequences of A5. PCR was performed in a volume of 50 μl containing 1 μl of the adaptor-ligated DNA digests (50 ng), a pair of primers (0.2 μM each), 1 U of Elongase (Invitrogen, Carlsbad, CA) and the reaction buffer as recommended by the supplier. The reaction mixture was incubated for 2 min at 94 °C, followed by 30 cycles: 1 min at 94 °C, 1 min at 55 °C and 10 min at 68 °C. The resulting products were electrophoresed on 1% agarose gels and extracted from the gels. The cloning of these DNA fragments was facilitated by using TA-cloning kit (Invitrogen). Plasmids were recovered using a QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA) and sequenced. The isolated sequences were analyzed by using the BLASTn program, National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/).

Reverse transcriptase (RT) PCR

Total RNA was extracted from a normal placenta of one SPF cat, independent tumor tissues of FeLV-induced thymic lymphoma in six cats which was challenged by an FeLV-A Rickard strain preparation, thymuses of two SPF cats, two feline normal T-cell lines of MCH5-4 and 104-C1 (Lerner et al., 1998), and three FeLV-related T-lymphoid tumor cell lines of FL-74, FT-1 and KO-1 using Trizol reagent (Invitrogen). After removal of DNA contamination using DNase I, 0.5 μg of total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen) in the presence of oligo(dT). Primers to amplify the partial fragment of activin-A receptor type II-like 1 (*ACVRL1/ALK1*) cDNA were designed based on the sequences of human and mouse homologs (GenBank/EMBL/DDBJ accession number BC042637 for human and CT010330, BC014291 and BC015083 for mouse). The reaction for the feline house-keeping *GAPDH* gene (GenBank/EMBL/DDBJ accession number AB038241) was used as an internal control. The primers were designed based on the identified sequence to cross an intron as follows: sense primer (5′ GCTT-CATCGCCTCAGACAT 3′) and antisense primer (5′ CAATGTGCTGGT-CAAGAGCAAC 3′) for *ACVRL1* cDNA, and sense primer (5′ CTCATGACCACAGTCCATGC 3′) and antisense primer (5′ TGAGCTTGA-CAAAGTGGTCA 3′) for *GAPDH* cDNA. PCR was performed as described above. The reaction mixture was incubated for 1 min at 94 °C, followed by 30 cycles: 1 min at 94 °C, 30 s at 55 °C and 1 min at 68 °C. The amplified products were electrophoresed on 2% agarose gels and visualized by staining with ethidium bromide. To confirm the sequence, these DNA fragments were extracted from the gels, cloned and sequenced as described above.

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