*Svi*3: A provirus common integration site in *c-myc* in SL/Kh pre-B lymphomas

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Spontaneous pre-B lymphomas in SL/Kh mice occur by somatic acquisition of a provirus genome of endogenous murine leukemia virus (MuLV). Inverse PCR amplification and sequence analyses of a provirus and its host flanking fragment revealed a proviral insertion into *c-myc* in 3 out of 60 SL/Kh pre-B lymphomas, named *Svi*3 lymphomas (*SL/Kh virus integration site*-3). Southern blot analysis revealed that two lymphomas had clonal integration in *c-myc* exon 1 and the other, in the promoter region. In 2 out of 3 *Svi*3 lymphomas, a fusion transcript of provirus 3′ long terminal repeat and *c-myc* and a normal full-length *c-myc* transcript were obtained, but in one *Svi*3 lymphoma, only the normal transcript was obtained. All three *Svi*3 lymphomas had increased *c-myc* expression, producing normal 67-kDa c-Myc protein. *Svi*3 lymphomas had more mature phenotypes in the steps of early B-cell differentiation than *Svi*1 lymphomas, in which *c-myc* expression was indirectly up-regulated by provirus integration into *Stat5a*. (Cancer Sci 2003; 94: 791–795)

It has been shown that retroviral insertions in host DNA may cause hematopoietic malignancies by enhancing the expression of a proto-oncogene or by disrupting tumor suppressor genes at the integration sites.[1)] Recent progress in the technique of retrotransposon tagging has greatly facilitated the identification of cancer-related genes.[2)]

SL/Kh, a pre-B lymphoma-prone inbred mouse strain, provides a unique model to investigate the host genetic and epigenetic factors determining susceptibility and types of disease.[3)] Mice of this strain develop spontaneous pre-B lymphomas at >90% incidence by 6 months of age.[4)] Several lines of evidence indicate that the endogenous murine leukemia virus (MuLV) plays an etiologic role in the genesis of SL/Kh lymphoma.[5–7)] Somatically acquired proviruses are frequently observed in the genomic DNAs of lymphomas.[6)] By applying inverse polymerase chain reaction (IPCR), we have identified a number of virus integration hot spots in SL/Kh lymphoma DNA; we have named them *SL/Kh virus integration sites (Svi)* and numbered them serially in the order of discovery.[7)] *Svi*3 is one of them, in which 3 out of 60 SL/Kh lymphomas had clonal integration of a provirus of the ecotropic MuLV within the promoter region and the first exon of the *c-myc*. The *c-myc* encodes a transcription factor that is a cellular homologue of the viral transforming gene encoded by the avian myelocytomatosis virus MC29.[8)] Leukemias and lymphomas induced by retroviruses frequently have integration in or near the *c-myc* gene,[9, 10)] suggesting that these tumors are clonal or semi-clonal outgrowths of cells that acquired a selective growth advantage *in vivo* as a consequence of *c-myc* insertions. In this report, we will describe the provirus integrations at *Svi*3 and their biological effects in SL/Kh mouse lymphomagenesis.

Materials and Methods

Mouse strain. SL/Kh is an inbred mouse strain maintained in the Department of Pathology and Biology of Diseases at the Kyoto University Graduate School of Medicine. Its origin,[11)] endogenous MuLV expression,[12)] pathology of lymphomas,[4, 12)] and role of host genetic factors in lymphomagenesis[5, 6, 13)] have been described elsewhere. Out of 60 primary SL/Kh lymphomas, 3 lymphomas, Nos. 110, 127, and 139, were found to have *Svi*3 integration. Lymphoma No. 126 was used as a reference tumor without virus integration into any of the known *Svi* loci, but with a typical phenotype of the SL/Kh pre-B lymphomas.

IPCR. Genomic DNA (100 ng) from each lymphoma was first digested with *Sac*II for 2 h and self-ligated with T4 ligase (TaKaRa, Otsu) at 14°C overnight. The virus-host junctions were amplified in a 50 μl reaction mixture containing 2.5 m*M* dNTP, 10 pmol/μl primer, and 0.25 unit of *Taq* polymerase (Expand Long Template PCR System) (Roche Diagnostics, Mannheim, Germany).

PCR amplification was carried out in three steps under the following conditions in a thermal cycler (PE Biosystems, Norwalk, CT): the first step was 10 cycles (each of 30 s at 94°C, 40 s at 62°C, and 4 min at 68°C) preceded by an initial denaturing step (1 min at 95°C); the second step was 20 cycles (each 30 s at 94°C, 40 s at 62°C, and 4 min plus 20 s extension at each successive cycle at 68°C); and the final step was elongation (10 min at 72°C).

The primers for IPCR were located within the Akv-MuLV genomes. Their sequences were 5B4, GAG GGC TTG GAC CTC TCG TCT CCT AAA AAA CCA CG and 5F1, GTC TCT CCC AAA CTC TCC CCC TCT CCA ACC in the first step; and 5F2, CCT CCT CTG ACG GAG ATG GCG ACA GAG AAG AGG and 5B1, GAG GGC TTG GAC CTC TCG TCT CCT AAA AGA ACC ACG in the second step of cycles for the nested PCR. The PCR products were electrophoresed in 1% agarose gel and stained with ethidium bromide. They were then subcloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA), and sequence analyses were performed using "BigDye" Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems).

Southern hybridization. Genomic DNA was extracted from independent lymphomas and the kidney of a newborn SL/Kh mouse kidney. A 10-μg sample of each tumor DNA was digested with a restriction enzyme, either *Nco*I, *Sac*I, *Sac*II, *Pvu*II, or *Hin*dIII (New England Biolabs, Beverly, MA) for 16 h and separated by electrophoresis on 1% agarose gels. The fragments were then transferred onto a N^+Hybond nylon membrane (Amersham, Piscataway, NJ). The membranes were incubated in a hybridization buffer[14)] at 65°C for 30 min. After boiling, a ^{32}P-labeled probe (10^6 cpm/ml; "Rediprime" II Random Prime Labeling System, Amersham) was added to the hybridization buffer, and the membranes were incubated in this mixture for 16 h at 65°C. The membranes were then washed once for 10 min at 65°C in 3× SSC+0.1% SDS and twice for 10 min each at 65°C in 0.1× SSC+0.1% SDS. Hybridization images were detected using Kodak Biomax MS Film (Eastman Kodak Co., Rochester, NY).

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DNA probes. The *c-myc* promoter probe (probe A) was prepared as described previously.[15] The *pSVcmyc1* was commercially obtained from the American Type Culture Collection (ATCC), Manassas, VA. A 4.8 kb insert for the probe (probe B) was extracted according to the manufacturer's protocol.

Northern blot analysis, 3′ rapid amplification of cDNA ends (RACE), and reverse transcription-PCR (RT-PCR). Total RNA was extracted from lymphoma tissues by ISOGEN (Nippon Gene, Toyama). The first-strand cDNA synthesis was performed using the "*Ready-To-Go*" T-Primed First-Strand Kit (Amersham). Northern blot analysis was performed as reported previously.[7] A 3′ RACE assay was performed with a 3′-Full RACE Core Set (TaKaRa). Upstream specific primers were TCC GAA TCG TGG TCT CGC TGA TCC TTG G, located in the provirus, and ACC CCT GGC TGC GCT GCT CT, located in *c-myc*. The 3′ primer was supplied with the kit.

The RT-PCR was performed as described previously.[16] The primers for β-actin were also prepared as reported previously.[16] The primers for other phenotype markers were as follows: *Ig*μ, TTC TGC CTG GTG ACA TTCC and TGG CTG TGT CAT TAG CTT GC; *VpreB*, ACG TCT GTC CTG CTC ATGC and TCT CCT TCC CAC TCT CTC TCC; *λ5*, TGA AGT TCT CCT CCT GCT GC and ACT ACG TGT GGC CTT GTT GG; *CD43*, CCA CCA CTG TGA CAA CAA GC and TTG TGG CCT CTT CAT CAGG; *CD79a*, ATC ACA TGG TGG TTC AGCC and TCT CCA ATG TGG AGG TTGC; *Blk*, CAG GCA GTC TCT CAA GTT GC and TCA TTG TAC AAC TCC GGT GG; *Syk*, GAG ATG AAT CAG AGC AGA CGG and GAT CAT GCG CAC AAT GTA GG.

Antibodies. Rabbit polyclonal antibodies to c-Myc (C-19), c-Myc (N-262), Max (C-124), and Topo I (C-15) and horseradish peroxidase (HRP)-conjugated antibodies to rabbit as well as goat immunoglobulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-actin monoclonal antibody was obtained from CHEMICON International, Inc. (Temecula, CA).

Cell culture and western blotting. NIH3T3-3 cells were obtained from the RIKEN Cell Bank (Tsukuba). They were maintained in Dulbecco's modified E4 medium (DMEM) supplemented with 10% fetal calf serum (FCS). The whole-cell lysate of NIH3T3-3 and nucleus as well as cytoplasm fractions from individual lymphomas were extracted as described previously.[7, 17] Twenty micrograms protein was separated by 7.5% SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membrane was incubated serially with primary and secondary antibodies (1/1000 dilution) dissolved in 1% Milk/TBS-T for 1 h each at room temperature. The bands were visualized by means of the chemiluminescence system ("ECL" Western Blotting Detection Reagents, Amersham).

Flow cytometry. The single-cell suspension (10^6 cells/ml) was prepared from lymph nodes of primary lymphomas. The cells were doubly stained with FITC-labeled anti-BP-1 (clone 6C3; PharMingen, San Diego, CA) and phycoerythrin-labeled anti-B220 (clone RA3-6B2; PharMingen) and analyzed in a FACScan (Becton Dickinson, San Jose, CA).

Results

Proviral integration into the *c-myc* locus. The SL/Kh lymphoma genomic DNA contains one or more copies of a somatically acquired provirus in addition to 7 copies of endogenous ecotropic MuLV proviruses.[5] Amplifying virus-host junctional fragments by IPCR from the genomic DNAs of 60 independent primary lymphomas, we found a case of integration into the 5′ promoter region and 2 cases of integration into the exon 1 of *c-myc* (Fig. 1A). These integration sites were collectively identified as the *SL/Kh virus integration-3 (Svi3)* locus. The viruses integrated in *Svi3* were in the direction of transcription and had a structure shared by most endogenous ecotropic MuLVs without a viral oncogene (data not shown). Southern hybridization of *Hin*dIII-digested DNAs with a *c-myc* promoter probe (probe A) and a 4.8 kb insert of *pSVcmyc1* (probe B) revealed that *c-myc* was rearranged in lymphomas Nos. 139, 110, and 127, but not in the non-*Svi3* lymphoma No. 126. Hybridization with probe A showed rearranged 3.8 and 6.3 kb bands in No. 139 lymphoma in addition to 1.3 and 0.8 kb germline bands seen in newborn kidney DNA and in No. 126 (Fig. 1B). In Nos. 110 and 127, hybridization with probe B revealed a 9.0 kb rearranged band in addition to 4.3, 1.3 and 1.1 kb germline bands. The rearranged 9.0 kb bands were less dense than the germline bands. In these cases, it is possible that the population with rearranged *c-myc* was not predominant, but might be oligoclonal or associated with numerous host cells. No rearranged band was seen in No. 126 lymphoma with probe B. The lymphomas with clonal integration to the *c-myc* locus were named *Svi3* lymphomas. The sequences of the provirus integration sites are shown in Fig. 1C.

Expression of c-Myc in *Svi3* lymphomas. Subsequently, the *c-myc* transcripts in individual *Svi3* lymphomas were examined by a 3′ RACE assay. The forward primers were set within the proviral long terminal repeat (LTR) region or within the first exon of *c-myc*. From Nos. 110 and 127 lymphomas, in addition to a full-length c-myc product, fusion transcripts consisting of

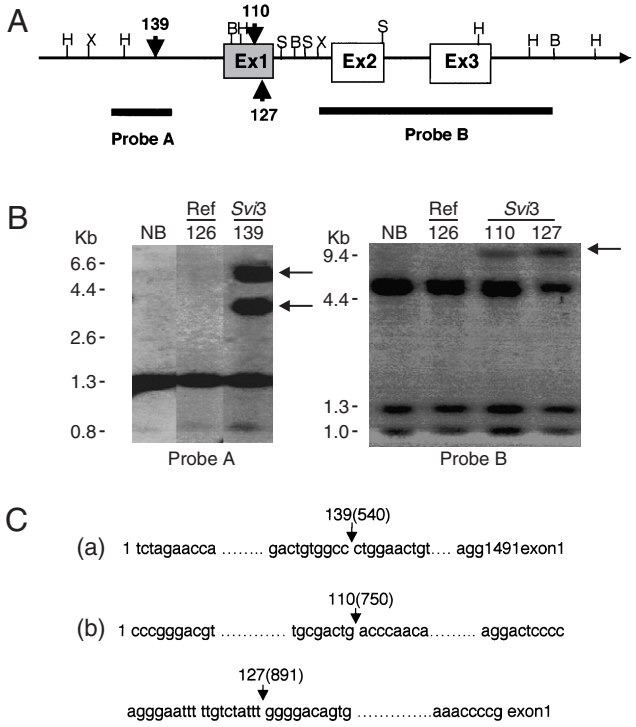

Fig. 1. Provirus integration in the *Svi3* hot spot. (A) Integration of proviruses in *Svi3* lymphomas in the promoter region of *c-myc* and to the first exon. The proviruses are indicated by arrowheads. Restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; B, *Bam*HI; S, *Sac*II. (B) Southern blot of *Hin*dIII-digested genomic DNA hybridized with either the *c-myc* promoter probe (probe A) or a 4.8 kb insert of the *pSVcmyc1* probe (probe B). NB, kidney of a newborn SL/Kh mouse; No. 126, a reference lymphoma; Nos. 110, 127, and 139, *Svi3* lymphomas. An arrow shows the rearranged band. Note that the MuLV provirus has an internal *Hin*dIII site. (C) Sequences of integration sites in *Svi3* lymphomas. (a) *c-myc* promoter region (the gene bank access number, MUSMYCN M12345). (b) *c-myc* exon1 (MUSCMYC1 L00038). The arrows indicate the integration sites of each lymphoma, and the sequence numbers are shown in parentheses.

retroviral LTR and c-myc mRNA initiated from the individual integration sites were obtained. On the other hand, from lymphoma No. 139, only the full-length transcript of *c-myc* was obtained. These transcripts are schematically shown in Fig. 2. Sequencing of the transcripts showed that the coding region of each transcript was intact and that no point mutation had occurred within them.

Northern blotting of the total RNA from lymphomas with probe B showed that *c-myc* expression was 3–5 times higher in *Svi*3 lymphomas than in the reference lymphomas (Fig. 3A). Western blotting with antibodies to the C- and N-terminal of c-Myc (C19 and N262) demonstrated increased 67-kDa c-Myc protein abundantly in nuclear fractions of *Svi*3 lymphomas (Fig. 3B), but scarcely in the cytoplasmic fraction (Fig. 3C). It seems that the elevated c-Myc was rapidly translocated into the nucleus immediately after synthesis in the cytoplasm.

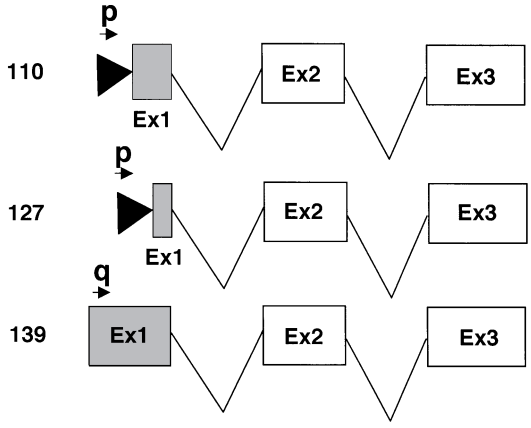

Fig. 2. Transcripts of *Svi*3 lymphomas. In Nos. 110 and 127, the transcript was a fusion product of a fragment of *c-myc* exon 1 fused with provirus 3′ LTR. In No. 139, a full-length *c-myc* transcript was obtained. Symbols p and q represent upstream specific primers used in the 3′ RACE assay analysis. Arrowheads indicate the proviruses.

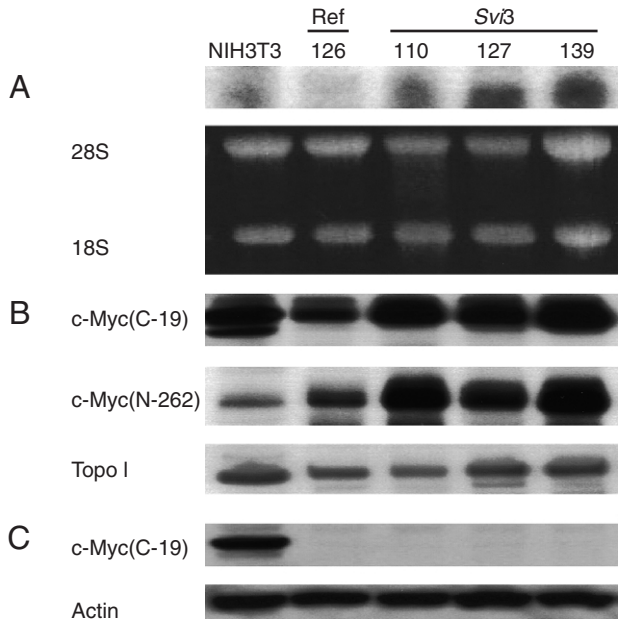

Fig. 3. Expression of *c-myc* in *Svi*3 lymphomas. NIH3T3, positive control; No. 126, reference lymphoma; Nos. 110, 127, and 139, *Svi*3 lymphomas. (A) Northern blotting with probe B. Total RNA, a loading control. (B) Western blotting of nuclear proteins with anti-c-Myc antibody (C-19 and N-262) and Anti-Topo I (as a loading control). (C) Western blotting of cytoplasmic proteins with an anti-c-Myc (C-19) antibody. NIH3T3 whole lysate, a positive control; anti-actin antibody, a loading control.

The phenotype of *Svi*3 lymphomas. FACScan analysis revealed that the *Svi*3 lymphoma was composed of BP-1 and B220 double-positive cells (Fig. 4A), as in most SL/Kh lymphomas.[3, 18, 19] We compared the phenotype of lymphomas with the integration in *Svi*1 (the second intron of Stat5a) and in *Svi*3. As previously shown, *Svi*1 lymphomas had elevated *c-myc* expression, since *c-myc* is one of the downstream transcription targets of *stat5a*.[7] RT-PCR analysis revealed a notable difference in an *Ig heavy chain (IgH)* μ gene and VpreB expression. They were positive in *Svi*3 lymphomas and in the reference tumor, but not in *Svi*1 lymphomas. Other genes important in early B-cell differentiation, i.e., $\lambda 5$, *CD43*, and *CD79a*, did not show a consistent difference between *Svi*1 and *Svi*3 lymphomas. Both lymphomas expressed protein kinase Syk and Blk. These phenotypes indicated that most SL/Kh lymphomas were in the pre-B stage, but there were subtle differences in differentiation among them, even when elevated *c-myc* expression was involved in lymphomagenesis. Lower expression of CD43 in the reference tumor No. 126 indicated that it may be a more mature form of pre-B lymphoma.

Discussion

Spontaneous pre-B lymphomas in SL/Kh mice occur by somatic acquisition of the provirus genome of the endogenous MuLV.[5–7] We have identified a number of virus integration hot spots in independent SL/Kh lymphoma DNAs.[7] One of them was the *Svi*3 locus, an approximately 2.0 kb stretch including the promoter region and first exon of *c-myc*.

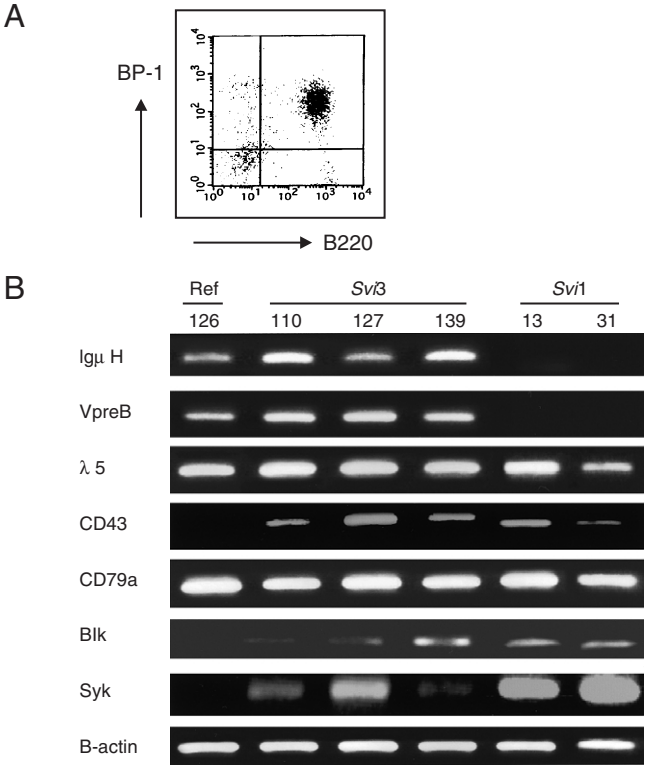

Fig. 4. The phenotype of *Svi*3 lymphomas. Flow cytometry of an *Svi*3 lymphoma showing double expression of BP-1 and B220. (B) Comparison of expressions of *Igμ*, *VpreB*, *λ5*, *CD43*, *CD79a*, *Blk*, and *Syk* in *Svi*1, *Svi*3 and reference lymphoma. β-Actin, a positive control. RT-PCR.

Activation of *c-myc* is most typically seen in human Burkitt's lymphomas, in which the *c-myc* exons 2 and 3 are juxtaposed with an *IgH* gene by translocation.[20] In *E*μ-*myc* (*IgH enhancer-myc*) transgenic mice, the development of B- and T-cell lymphomas was dependent on the mouse strain.[21, 22] In mouse spontaneous lymphomas, however, a provirus insertion into *c-myc* is frequently found in T-cell lymphomas[23, 24] but relatively rarely in B-cell lymphomas[25] except for those in some AKXD recombinant inbred strains,[26] *Cdkn2a*-targeted C57BL mice injected with Moloney MuLV[27] and athymic mice infected with MuLV.[28]

The prevalence of pre-B lymphomas in SL/Kh mice may be explained by their host genetic predisposition. The SL/Kh mice show transient expansion of pre-B cells in bone marrow at 3–6 weeks of age in advance of lymphoma development.[3, 18, 19] Such an expansion is not caused by MuLV[18, 29, 30] but by a semi-dominant SL/Kh allele at *Bomb1* on chromosome 3.[13] The inherited abnormality in the development of the B-cell lineage, therefore, seems to amplify the target of viral oncogenesis. SL/Kh mice have other genes favoring pre-B lymphoma development. In the cross between SL/Kh and AKR, the predominant tumors are T-lymphomas in individuals bearing a dominant AKR-derived allele at *Tlsm1*.[31] On the other hand, in the cross between SL/Kh and NFS/N, a pre-B lymphoma developed only in individuals with a dominant SL/Kh allele at *Esl1*, an *MHC*-linked locus on chromosome 17.[6] An accumulation of these genetic factors may predispose SL/Kh mice to pre-B lymphomas.

The types of MuLVs involved in lymphomagenesis may well be another determinant. Sequence analysis of the proviral genomes in *Svi*3 showed that they had the general structure of Akv and other endogenous ecotropic MuLV, except for an internal *Hin*dIII site shared with SL3-3 MuLV. The SL/Kh mice are highly viremic for both eco- and xenotropic MuLV, but the thymotropic mink cell focus-forming virus is not detected.[12] Inoculation of lymphoma extracts into NFS newborns induces nodular hyperplasia or follicular lymphomas in the spleen after 15 months or later (Hiai, unpublished observation). A similar observation was reported for NIH-Swiss mice congenic for *Akv1* and *Akv2*.[32]

We found an LTR-*c-myc* fusion transcript in 2 out of 3 *Svi*3 lymphomas. Such a fusion transcript has been reported in avian leukemia virus[33] and MuLV-induced T-cell lymphomas,[34] but not in murine T-cell lymphomas induced by Moloney MuLV or mink cell focus-forming virus.[23, 24] Even in the *Svi*3 lymphomas with the fusion transcript, a normal 67-kDa c-Myc protein was obtained. This observation again suggests that an intact c-Myc protein is required for induction of leukemias and lymphomas during insertional mutagenesis by retrovirus.[25]

Finally, we examined whether or not the mode of *c-myc* activation affects the phenotypes of the lymphomas by comparing the phenotype of *Svi*3 lymphomas with that of *Svi*1 lymphomas. As previously reported,[7] *Svi*1 lymphomas had elevated *c-myc* expression, since activated *stat5a* could target *c-myc* via binding to a GAS element of the *c-myc* promoter. It is well documented that pre-B cell development is highly dependent on the preBCR signal pathway, in which an *Src* family protein tyrosine kinase Blk and an Syk/ZAP70 family protein tyrosine kinase Syk are recruited to preBCR following its activation.[35] We found that *Blk* and *Syk* were highly expressed in both *Svi*1 and *Svi*3, but only slightly expressed in the majority of SL/Kh lymphomas, including the reference lymphoma. This implies that the *Svi*3 and *Svi*1 lymphomas probably share the pre-B cell receptor signaling pathway. We found expression of the *Ig*μ gene and *VpreB* in *Svi*3 and reference lymphomas, but not in *Svi*1 lymphomas. This suggests that *Svi*3 lymphomas were more mature than *Svi*1 lymphomas. Both *Svi*3 and *Svi*1 lymphomas were positive for CD43, indicating that these lymphomas were more immature than the reference tumor without CD43 expression. Judged from their phenotypes, the order of differentiation among SL/Kh lymphomas is *Svi*1, *Svi*3 lymphomas, and the reference lymphoma. The phenotype of *Svi*3 lymphomas with direct activation of *c-myc* by provirus integration was, therefore, fixed at a later stage in early B-cell differentiation than that of *Svi*1 lymphomas with indirect up-regulation of *c-myc*.

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