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GFI1B, EVI5, MYB—Additional genes that cooperate with the human *BCL6* gene to promote the development of lymphomas

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Abstract

The *BCL6* gene, which is expressed in certain B- and T-cell human lymphomas, is involved with chromosomal rearrangements and mutations in a number of these neoplasms. Lymphomagenesis is believed to evolve through a multi-step accumulation of genetic alterations in these tumors. We used retroviral insertional mutagenesis in transgenic mice expressing the human *BCL6* transgene in order to identify genes that cooperate with *BCL6* during lymphomatous transformation. We previously reported *PIM1* as the most frequently recurring cooperating gene in this model. We now report three newly identified cooperating genes—*GFI1B*, *EVI5*, and *MYB*—that we identified in the lymphomas of retroviral-injected *BCL6* transgenic mice (but not in retroviral-injected non-transgenic controls); mRNA and protein expression of GFI1B and EVI5 were decreased in the murine tumors, whereas MYB mRNA and protein expression were increased or decreased. These findings correlated with protein expression in human lymphomas, both B- and T-cell. Improved therapy of lymphomas may necessitate the development of combinations of drugs that target the alterations specific to each neoplasm.

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All authors declare no conflict of interests.

Declaration
The work has not been published previously and is not under consideration for publication elsewhere. The work is approved by all authors. The work, if accepted, will not be published elsewhere, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

Keywords

BCL6 transgenic mice; Cooperating genes; Lymphoma development; GFI1B; EVI5; MYB

Introduction

It has long been known that chromosomal rearrangements and/or mutations of the *BCL6* gene are associated with diffuse large B-cell lymphomas (DLBLs) in humans [1]. BCL6 expression also is known to occur in certain T-cell lymphomas [2]. Because multiple oncogenic hits are believed to accumulate during lymphoma development, we used retroviral insertional mutagenesis in mice containing the human *BCL6* transgene in an effort to identify mutated genes that cooperate with *BCL6* during lymphoma development [3].

We previously reported that retroviral insertional mutagenesis in mice transgenic for human *BCL6* leads to enhanced lymphoma development, and we described the gene proviral integration site for Moloney murine leukemia virus 1 (*PIM1*) as the most frequently recurring gene cooperating with *BCL6* to promote lymphoma development in this model [3]. We now report additional recurring retroviral integration sites that we identified in the lymphomas which developed in retroviral-injected *BCL6* transgenic mice but not in lymphomas from retroviral-injected non-transgenic controls. The mRNA and proteins encoded by two of the genes involved, growth factor independent 1B (*GFI1B*) and ecotropic viral integration site 5 (*EVI5*), each of which we identified in the lymphomas from three transgenic animals (three B-cell and three T-cell, respectively), showed decreased expression in the lymphomas from transgenic (BCL6-positive) mice as compared with the lymphomas from non-transgenic retroviral injected controls (BCL6-negative) as well as in BCL6-positive (as compared with BCL6-negative) human lymphomas. The mRNA and protein encoded by a third involved gene, myeloblastosis oncogene (*MYB*), which we also identified in the lymphomas from three transgenic animals (two B-cell, one T-cell) but not in controls, was variably increased or decreased. Although these three genes have been associated with lymphoma development [4–6], they have not been reported previously to cooperate with *BCL6* in lymphomagenesis.

Materials and methods

BCL6 transgenic mice and retroviral insertional mutagenesis

Our transgenic mice (described previously) [7], which express BCL6 constitutively in B- and T-lymphocytes, contain two transgenes: tet-o-BCL6 (human BCL6 cDNA under control of the tetracycline-responsive minimal promoter), and EμSR-tTA (the tetracycline-transactivating protein under control of the Ig heavy-chain enhancer and the SRα promoter). One of our transgenic lines contains four copies of the human *BCL6* transgene, the other 20 copies. Under approved institutional protocols, neonatal animals from both transgenic lines and controls (non-transgenic mice of the same background, wild-type or positive for either the EμSR-tTA or tet-o-BCL6 transgenes, but not both) were injected with 10^5 infectious units of the retrovirus MOL4070LTR [3] intraperitoneally and followed until the need for euthanasia or natural death.

Histology, flow cytometry, inverse PCR, database searches, and real-time quantitative PCR

Processing of tissues, FACS analysis, inverse PCR, cloning, sequencing, database searches, total RNA extraction, and real-time RT-PCR methodologies have been described previously [3]. The classification of murine lymphoid neoplasms was according to the Bethesda proposals [8]. Primers included β-actin as described [3] or glyceraldehyde-3-phosphate dehydrogenase (Real Time Primers, Elkins Park, PA) and primers common to all transcript variants of the murine *GFI1B*, *EVI5*, or *MYB* genes (*GFI1B*, forward: 5′-CTCTCCAGGCATGGACACTT-3′; reverse: 5′-GACGTGAGTATGCTGCTCCA-3′; *EVI5*, forward: 5′-CCCATCAAAGTTG AGTCCAG-3′; reverse: 5′-TTCTTCCCCAGAGAATCCAA-3′; *MYB*, Real Time Primers, forward: 5′-CTGGACAGAAGAGGAGGACA-3′; reverse: 5′-TTGTT CTTCTGGAAGCTCGT-3′).

Immunohistochemistry

Human lymphoma blocks were retrieved from the surgical pathology archives under an Institutional Review-Board approved protocol. Human and murine tissues were stained with anti-BCL6 as described previously [3]. For additional immunohistochemistry, antigen retrieval was performed in pH 6 solution (Epitomics, Burlingame, CA) in a near boiling waterbath for 40 min. Anti-GFI1B (#HPA007012, for human tissues) and anti-EVI5 (#HPA027339, for human and murine tissues) were affinity-isolated Prestige antibodies produced in rabbit (Sigma-Aldrich, Saint Louis, MO). Anti-GFI1B was diluted 1:20 and anti-EVI5 1:35 for incubation overnight at 4 °C. Murine tissues were stained with GFI1B affinity isolated antibody produced in rabbit (Sigma Aldrich, #AV30093), 20 μg/ml, overnight at 4 °C. For immunohistochemistry with MYB antibody, human tissues were incubated overnight at 4 °C with a 1:20 dilution of a rabbit monoclonal antibody (Epitomics, Burlingame, CA, cat. # 3195-1). Murine tissues were incubated with the same MYB antibody diluted 1:1000 for 1 h at room temperature. For all antibodies, antigen-antibody binding was detected with DAB chromogen, and tissues were counterstained with hematoxylin. Images were taken with a BX41 microscope (Olympus), a DP72 digital camera, and cellSens Standard imaging software (Olympus).

Statistical analysis

The data from real-time PCR were analyzed by the comparative C_T method [9]. Data from the study and control groups were compared by the Wilcoxon rank-sum test. Statistical analyses were performed with Stata software (version 12: StataCorp, College Station, TX).

Results

Lymphoma development in MOL4070LTR-infected mice

As previously reported, 92 of 99 transgenic and 32 of 33 control mice infected with retrovirus were analyzable [3]. Lymphomas developed in 24 of 53 animals (45.3%) in the four copy transgenic line (18 T-cell, six B-cell) and in 10 of 39 (25.6%) mice from the 20 copy line (three T-cell, seven B-cell). Additionally, as MOL4070LTR is known to cause lymphomas and leukemias in mice [10], lymphoid neoplasms also were expected in the control group; 11 of 32 retroviral-injected controls (34.4%) developed lymphomas (5 T-cell,

6 B-cell). The nature of the lymphomas (both B- and T-cell), mouse survival, and murine lymphoma-related mortality rates have been discussed previously [3]. Briefly, the lymphomas usually were aggressive tumors; most of the B-cell lymphomas were mature B-cell, and most T-cell tumors were precursor T-cell lymphoblastic lymphoma/leukemias. The lymphomas from the transgenic mice contained three to 29 insertion sites (average 11), and the lymphomas from the controls contained eight to 20 insertion sites (average 12.7) [3]. Only one of the animals studied here (with an insertion site 5′ to the *GFI1B* gene) also had an insertion site 3′ to *PIM1*.

RNA expression in lymphomas containing retroviral insertions in or near the GFI1B, EVI5, and MYB genes

As previously described, *PIM1* was the gene most commonly affected by viral insertion sites in this study [3]. Here we have chosen to study the genes that were the next most commonly affected by viral insertions (after *PIM1*). The types of lymphomas, insert locations, and relative expression levels following real-time quantitative RT-PCR performed on RNA derived from the lymphomas of transgenic mice as compared with RNA from lymphomas of the same type (B or T) in the same organ from multiple randomly selected non-transgenic controls are shown in Table 1.

The insertion sites near *GFI1B* were all within 7.9–8.7 kb 5′ of the *GFI1B* gene, and these murine tumors were all large B-cell lymphomas. The transgenic animals were females, ranging in age from 4.2 mos to 7.3 mos (average, 6.2 mos), and included both *BCL6* transgenic lines. In each case, relative *GFI1B* RNA expression level in the lymphomas from transgenic mice was decreased as compared with that in randomly selected B-cell lymphomas from retroviral-injected non-transgenic controls (none of which had inserts in or near *GFI1B*), see Table 1 (overall decrease, 2.71-fold; mean ± SEM = 0.041 ± 0.018 in the controls as compared with 0.015 ± 0.005 in the transgenics; $P < 0.05$, Fig. 1a).

In contrast, the insertion sites involving *EVI5* all occurred in T-cell tumors (precursor T-cell lymphoblastic lymphoma/leukemias) from the four copy *BCL6* transgenic line. One of these insertions was in the terminal intron, and two were in the 3′ untranslated region of the *EVI5* gene. One mouse was female, two were males, and these animals ranged in age from 3.8 mos to 5.9 mos (average, 4.6 mos). Relative EVI5 RNA expression levels were decreased in the T-cell lymphomas from the transgenic mice as compared with T-cell tumors from randomly selected controls, none of which had inserts in or near *EVI5* (overall decrease, 1.86-fold; mean ± SEM = 0.007 ± 0.001 in the controls as compared with 0.004 ± 0.0007 in the transgenics; $P < 0.05$, Fig. 2a).

The retroviral inserts near *MYB* were all 3′ (34.8 to 73.7 kb) and occurred in three females ranging in age from 4.7 mos to 7.8 mos (average, 6.6 mos). Two were large B-cell lymphomas from the 20 copy transgenic line and one was a precursor T-cell lymphoblastic lymphoma/leukemia from the four copy transgenic line. Relative RNA expression in one of the B-cell tumors and in the T-cell lymphoma was increased ≥2-fold as compared with that in the three randomly selected control B-cell lymphomas and two randomly selected control precursor T-cell lymphoblastic lymphoma/leukemias, respectively (which did not contain inserts in or near the *MYB* gene), but relative RNA expression in the other B-cell lymphoma

was 3.2-fold decreased as compared with that in the lymphomas from the three randomly selected B-cell controls. These data are depicted in Fig. 3a.

GFI1B, EVI5, and MYB protein levels in lymphomas from retroviral-injected BCL6 transgenic mice and controls, and in human BCL6-positive and negative lymphomas

Protein levels in murine lymphomas detected by immunohisto-chemistry correlated with RNA levels from murine tumors quantified by real-time PCR as described above, and human lymphomas had a similar staining pattern as the murine counterparts (Figs. 1b, 2b, 3b). Whereas GFI1B expression was detected in the cytoplasm, EVI5 and MYB had a nuclear localization.

Tissue sections from the lymphomas of the three transgenic mice containing retroviral inserts near *GFI1B* (all large B-cell) and from B-cell lymphomas of three randomly selected retroviral-injected nontransgenic controls (no control tumors contained inserts near *GFI1B*) were studied with anti-GFI1B. Representative sections, shown in Fig. 1b, panel I, indicate that GFI1B staining is decreased in transgenic tumors (nuclear staining is positive for BCL6) as compared with the tumors in non-transgenic retroviral-injected controls (nuclear staining is negative for BCL6). Additionally, sixteen human lymphomas were studied by immunohistochemistry for GFI1B. Nine were B-cell tumors, seven were T-cell neoplasms; eight were BCL6-positive (six B-cell, two T-cell), and eight were BCL6-negative (three B cell, five T-cell). GFI1B expression was decreased in the BCL6-positive lymphomas as compared with the BCL6-negative tumors, which showed strong cytoplasmic expression of GFI1B (Fig. 1b, panel II). Thus, the findings in human lymphomas paralleled those in mice. Additionally, whereas in mice inserts near *GFI1B* were noted only in BCL6-positive B-cell tumors (which demonstrated decreased expression of GFI1B RNA and protein levels as compared with BCL-negative murine lymphomas), in humans, BCL6-positive T-cell as well as B-cell neoplasms demonstrated decreased expression of GFI1B protein as compared with BCL-negative lymphomas of each cell type (Fig. 1b, panel II).

Representative immunohistochemical staining of the murine lymphomas containing retroviral inserts in the *EVI5* gene (all precursor T-cell lymphoblastic lymphoma/leukemias) is shown in Fig. 2b, panel I and reveals that EVI5 staining (nuclear) is decreased in the BCL6-positive T-cell neoplasms as compared with the non-transgenic retroviral-injected BCL-negative T-cell controls, none of which contained inserts in or near *EVI5*. Twenty-seven human lymphomas were studied with anti-BCL6. Eight were negative for BCL6 (3 B-cell, 5 T-cell), 17 were BCL6-positive (13 B, 4 T), and two (T-cell) stained weakly with anti-BCL6. Representative sections (Fig. 2b, panel II) indicate that BCL6-positive lymphomas contain less EVI5 than do BCL-negative neoplasms. As in the case of GFI1B, findings for EVI5 in human lymphomas paralleled the findings in murine lymphomas, and, additionally, whereas in the mouse tumors inserts in the *EVI5* gene were noted only in BCL6-positive T-cell tumors (which had decreased expression of EVI5 RNA and protein as compared with BCL-negative T-cell lymphomas), in human lymphomas, decreased levels of EVI5 protein were present in BCL6-positive B- as well as T-cell tumors as compared with BCL-negative controls (Fig. 2b, panel II). Non-neoplastic BCL6-positive germinal center

cells also contained less *EVI5* than the surrounding BCL-negative tumor cells (Fig. 2b, panel II, 4C).

Representative immunohistochemistry of MYB staining (Fig. 3b, I) of two murine lymphomas from transgenic mice (a B-cell tumor, panel 1 and a T-cell tumor, panel 2), reveals that these BCL6-positive neoplasms also show nuclear staining for MYB, whereas two murine lymphomas from non-transgenic animals (B-cell, panel 3, and T-cell, panel 4) are BCL-negative (column B) as well as MYB-negative (column C). A third transgenic mouse with a large B-cell lymphoma (not shown) showed little to no staining for *MYB*. Murine lymphomas stained readily with anti-MYB, which was used in a dilution of 1:1000 for 1 h at room temperature. Human lymphomas were more difficult to stain, requiring a 1:20 dilution and overnight incubation, and even then, positive staining was often focal and noted in only six (24%) of the 25 lymphomas studied. Of the 25 human lymphomas (12 B-cell, 13 T-cell) studied, 17 were BCL6-positive (10 B-cell, seven T-cell), five were BCL-negative (one B-cell, four T-cell), and three stained weakly for BCL6 (one B-cell, two T-cell). In four BCL6-positive lymphomas (two B-cell, two T-cell), the MYB-positive cells were also BCL6-positive (Fig. 3b, II, panels 1 and 2); however, in two T-cell lymphomas, one BCL6-positive, but with variably weak BCL6 staining, and one BCL-negative (Fig. 3b, panels 3 and 4, respectively), the MYB-positive cells were BCL-negative. Our observations in human lymphomas paralleled those in murine tumors in the respect that *MYB* levels could be increased or decreased in BCL6-positive neoplasms, T- as well as B-cell, and in human tumors, MYB positivity could be demonstrated in BCL6-positive as well as BCL-negative neoplastic cells (Fig. 3b, panel II).

Recurring sites in BCL6-positive transgenic mice not found in nontransgenic controls

Table 2 lists the genes nearest to recurring sites found in at least two transgenic animals but not in non-transgenic controls. Additional genes that may cooperate with *BCL6* occurred in multiple retroviral-injected transgenic mice and also in at least one retroviral-injected non-transgenic (control) animal (e.g., MYC), and thus may have transforming properties on their own. These are not reported here.

We previously reported that the most frequent retroviral insertions in our *BCL6* transgenic mice occurred in or near the *PIM1* gene, and we showed overall higher levels of *PIM1* RNA and protein (by immunohistochemistry) in lymphomas (B- or T-cell) containing these insertion sites [3]. Here we have studied the genes involved by the next most common insertion sites (in or near *GFI1B*, *EVI5*, and *MYB*, which were observed in lymphomas from three transgenic mice in each case).

Discussion

Although the *BCL6* gene is best known for its association with DLBLs in humans (~40% of these neoplasms are associated with chromosomal rearrangements involving *BCL6* and ~16% contain mutations disrupting autoregulation of the *BCL6* gene) [1], the role of BCL6 in T-cell development and function has gained increased attention [11,12]. Through the use of retroviral insertional mutagenesis in *BCL6* transgenic mice, we have identified three genes that have not been reported previously as cooperating with *BCL6* to promote

lymphomagenesis. All of these have been recognized as proto-oncogenes [13–15]. Two (*GFI1B* and *MYB*) are transcription factors [16,17] and the third (*EVI5*) is considered an essential regulator of cell membrane trafficking [18,19]. Additionally, immunohistochemistry performed on B- and T-cell human lymphomas (16 in the case of GFI1B, 27 for EVI5, 25 for MYB), both BCL6-positive and negative, confirmed our observations in murine lymphomas at the RNA and protein level: GFI1B and EVI5 are downregulated in BCL6-positive lymphomas as compared with BCL-negative tumors, and MYB can be either up- or down-regulated in BCL6-positive lymphomas as compared with BCL-negative controls.

The *GFI1B* gene, located on human chromosome 9q34.13, is not a common target in retroviral insertional mutagenesis [20]. It encodes a transcriptional repressor with an N-terminal SNAG (Snail/Gfi1) domain and six C-terminal zinc fingers that are believed to have an important role in hematopoiesis. Of two transcripts in humans, the most frequent is a 330 amino acid protein which binds DNA and suppresses gene expression through recruitment of histone modifying enzymes at target promoters [16]. It is expressed in myeloid progenitor cells as well as in B- and T-cell subsets [21], is known to control expression levels of genes critical for B-cell development [22], and is essential for erythroid and megakaryocytic development [21]. It is not expressed in mature thymocytes [23].

The *EVI5* gene, located on human chromosome 1p22, is a common viral integration site in T-cell lymphomas derived from AKXD mice [5]. It encodes a coiled-coil protein [5] that is considered essential for regulation of membrane trafficking [18,19] and has been believed to be involved in T-cell disease [5]. As we noted in our murine lymphomas, a common viral integration site is in a 3′ intron, and integrations are opposite to the transcriptional orientation of the gene [5]. Although real-time RT-PCR revealed decreased RNA expression in all of the lymphomas from the transgenic mice as compared with the nontransgenic controls, the differences were not large; however, it is known that even small perturbations in the expression level of a protein may lead to significant biological effects [24,25]. Alternatively, as it is known that gene expression can be affected over hundreds of kilobases by retroviral integrations [26], it is possible that viral insertions in the *EVI5* gene could affect another gene, e.g., growth factor independent 1 (*GFI1*), which is located 18 kb downstream from *EVI5* and is known to be a transcription factor that is crucial for normal hematopoietic development. Thus, Schmidt et al. [27], who could not detect a signal with an *EVI5* probe on Northern blots prepared from total RNA of lymphomas bearing proviral *EVI* integrations in *MYC*/*PIM* bitransgenic mice, found that the tumors had enhanced GFI1 RNA expression. However, these authors indicate that other targets which are located several hundred kilobases apart also could be activated by *EVI5* integrations.

MYB, a site of recurrent retroviral insertional mutagenesis in a number of murine hematopoietic malignancies [28], is a leucine zipper DNA-binding transcription factor [29] with a short half-life that undergoes post-translational modifications, including ubiquitylation, phosphorylation, acetylation, and sumoylation [30]. Human *MYB*, located on chromosome 6q23.3 [17], contains 15 exons and encodes a family of related proteins. Alternative splicing leads to splice variants that are predicted to encode proteins with differing transcriptional activities and specificity domains [31]. The MYB protein is

expressed in all proliferating hematopoietic cells, is involved in the regulation of proliferation and differentiation of bone marrow progenitor cells (also of colon and adult brain) [30,32], and is required for normal hematopoiesis, T-cell development, pro-B to pre-B transition, and survival of spleen B cells [30,33]. In humans, expression of MYB is known to be relevant for the lymph node germinal center phenotype, and that expression is sustained by BCL6 repression of microRNA (miR)-155 [34]. Although, traditionally, MYB has been touted as a transcriptional activator, several corepressor molecules that interact through various MYB domains imply that MYB could act also as a repressor [28]. In one study, about half of the genes found to be regulated by MYB were repressed [35]. Its target genes have functions in cell cycle progression, cell differentiation, and survival [30,32]. The gene is known to be translocated in T-cell acute lymphocytic leukemia, and its protein is overexpressed in that disease, in part through gene duplication [29].

In addition to the three genes described above, additional genes of potential interest are listed in Table 2. Almost all of these are known to be involved in hematopoiesis or hematologic malignancies, lending validity to the notion that they may well cooperate with *BCL6* in lymphomagenesis. For example, *LMO2* is up-regulated in non-Hodgkin lymphomas [36] and has been noted to be translocated in certain human T-cell acute lymphoblastic leukemias [37]; *MN1* is deregulated in human acute myeloid leukemia [38]; *AHI1* is an oncoprotein that interacts with BCR-ABL and Janus kinase 2 (JAK2) in chronic myelogenous leukemia cells [39] and is expressed in cutaneous T-cell lymphomas with intermediate to poor prognosis [40]; BCOR is part of a BCL6 repression complex that facilitates survival and proliferation of lymphomas [41]; *IKZF1* encodes the IKAROS transcription factor, which drives lymphoid development [42], and *IKZF1* deletions have been noted in childhood B-cell precursor acute lymphoblastic leukemia [43]; *SYK* was identified as a target in acute myeloid leukemia [44] and, when involved in translocations with the interleukin 2-inducible T cell kinase (*ITK*) gene, it induces a T-cell lymphoproliferative disease in mice mimicking human disease [45]; *DNTT* encodes a lymphoid regulator [46]; and, finally, *GATA1* encodes an erythroid transcription factor [47].

BCL6 is believed to exert its function by repressing hundreds of proteins [34]. B- as well as T-cell lymphomas are aggressive hemato-logic neoplasms resulting from the malignant transformation of B- or T-cell progenitors, respectively. These transformation processes are believed to be multi-step events in which a series of heterogeneous genetic alterations cooperate to induce perturbations in normal lymphocyte growth and differentiation. DLBLs [48] and T-cell acute lymphoblastic leukemias [29], for example, have been reported to harbor multiple genetic abnormalities (in the case of DLBLs, more than 30 clon-ally represented gene alterations per neoplasm) [48] which differ from tumor to tumor, making approaches to therapeutic intervention diffi-cult. Nonetheless, therapies directed against MYB [30] and the proteins encoded by a number of other genes known to be involved in lymphomatous transformation are currently under investigation. Our studies have added *GFI1B*, *EVI5*, and *MYB* to the list of genes cooperating with *BCL6* during lymphomagenesis, thus expanding the arsenal of potential therapeutic targets for DLBL. The finding in this study of several down-regulated cooperating genes suggests that efforts to upregulate them, e.g., by target mRNA manipulation [49], may have some therapeutic

benefit. Identification of the alterations in individual lymphomas, with selection of the appropriate combinations of agents targeting the specific lesions, may hold promise for therapy of these neoplasms.

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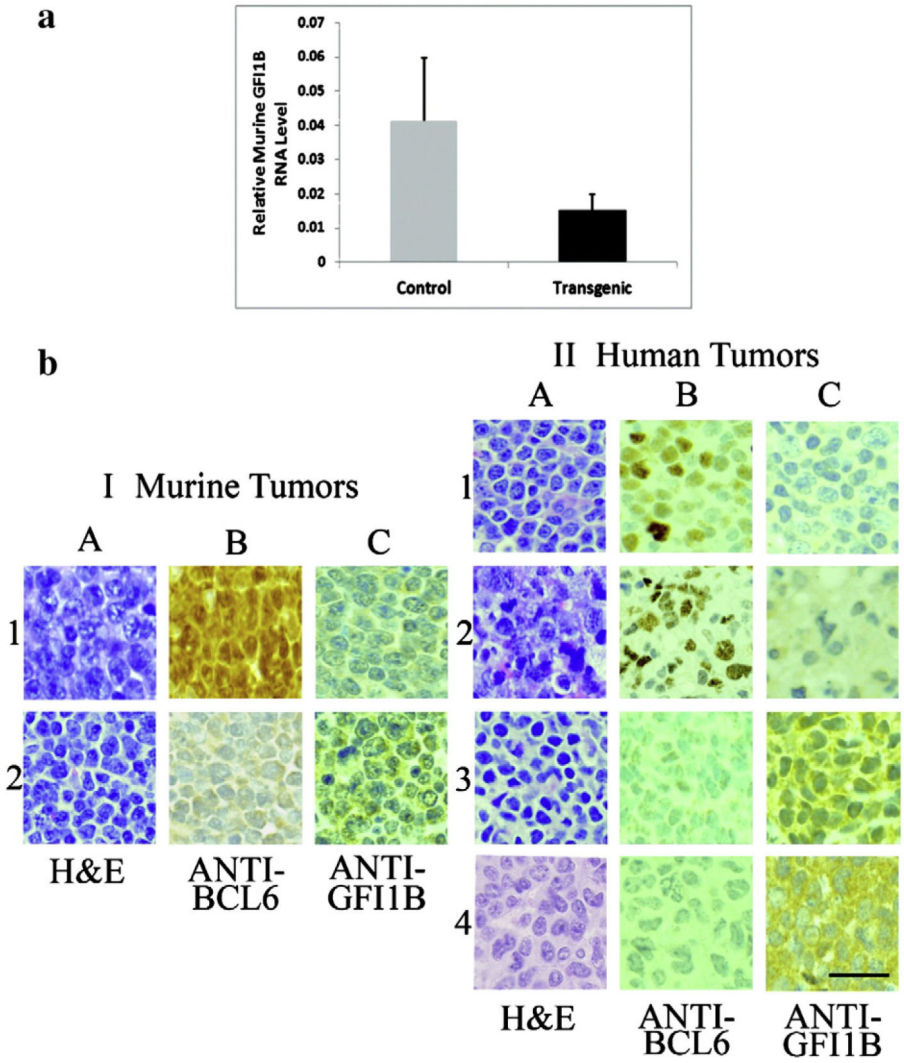

Fig. 1.

GFI1B: relative RNA levels in lymphomas from *BCL6* transgenic and control mice and immunohistochemistry of BCL6-positive and -negative murine and human lymphomas. (a) The graph depicts average relative GFI1B RNA expression from the lymphomas (all large B-cell) of three retroviral-injected *BCL6* transgenic mice (black bar) containing inserts 7.9 to 8.7 kb 5′ to the *GFI1B* gene as compared with three randomly selected B-cell lymphomas from retroviral-injected non-transgenic controls that did not contain inserts in or near *GFI1B* (gray bar). Expression in each transgenic animal was decreased as compared with the controls [range, 1.8 to 5.7-fold (Table 1), overall decrease 2.71-fold; mean ± SEM = 0.041 ± 0.018 in the controls as compared with 0.015 ± 0.005 in the transgenics; $P < 0.05$]. (b) I, Representative murine lymphomas from (1) a transgenic mouse (nuclei positive for BCL6, column B) and (2) a non-transgenic animal (nuclei negative for BCL6). The cytoplasmic staining, evident in the anti-GFI1B (C) column, is decreased in the transgenic mouse as compared with the non-transgenic control. II, Representative human lymphomas: (1) BCL6-positive (B-cell), (2) BCL6-positive (T-cell), (3) BCL6-negative (B-cell), (4) BCL6-negative (T-cell). As in the murine lymphomas, the GFI1B cytoplasmic staining (column C)

is decreased in the BCL6-positive lymphomas as compared with the stronger expression in the BCL6-negative neoplasms. The bar (lowest right panel) indicates 50 μm.

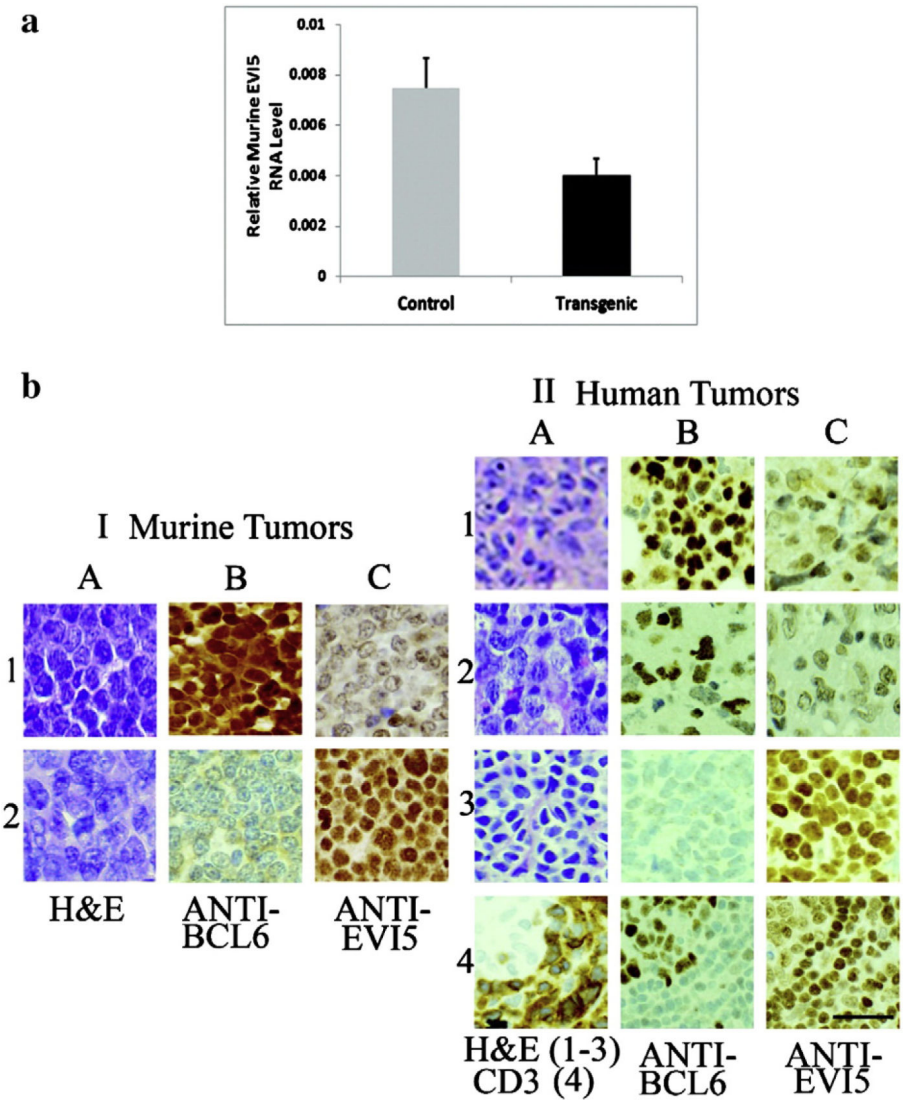

Fig. 2.

EVI5: relative RNA levels in lymphomas from BCL6 transgenic and control mice and immunohistochemistry of BCL6-positive and -negative murine and human lymphomas. (a) The graph depicts average relative EVI5 RNA expression from the lymphomas (all T-cell) of three retroviral-injected *BCL6* transgenic mice (black bar) containing inserts within the terminal intron or 3′-untranslated region of the *EVI5* gene as compared with three randomly selected T-cell lymphomas from retroviral-injected non-transgenic controls that did not contain inserts in or near *EVI5* (gray bar). Expression in each transgenic animal was decreased as compared with the controls [range, 1.3 to 2-fold (Table 1), overall decrease, 1.86-fold; mean ± SEM = 0.007 ± 0.001 in the controls as compared with 0.004 ± 0.0007 in the transgenics; $P < 0.05$]. (b) I, Representative murine lymphomas from (1) a transgenic mouse (nuclei positive for BCL6, column B) and (2) a non-transgenic mouse (nuclei negative for BCL6). The nuclear staining evident in the anti-BCL6 (C) column is decreased in the transgenic mouse as compared with the non-transgenic control. II, Representative human lymphomas: (1) BCL6-positive B-cell, (2) BCL6-positive (T-cell), (3) BCL-negative

(B-cell), (4) BCL-negative (T-cell); the cytoplasm of the tumor cells stains strongly positive with the T-cell marker CD3 (II, 4A). A germinal center in 4A (upper left corner) does not stain with CD3 and is BCL6-positive (column B), whereas the tumor cells around it are BCL-negative. As in the murine lymphomas, the EVI5 nuclear staining (column C) is decreased in BCL6-positive cells (tumors 1 and 2, or germinal center cells, tumor 4) as compared with the stronger expression in the BCL-negative tumor cells [tumors 3 and 4 (outside the germinal center)]. The length of the bar in the lowest right panel depicts 50 μm.

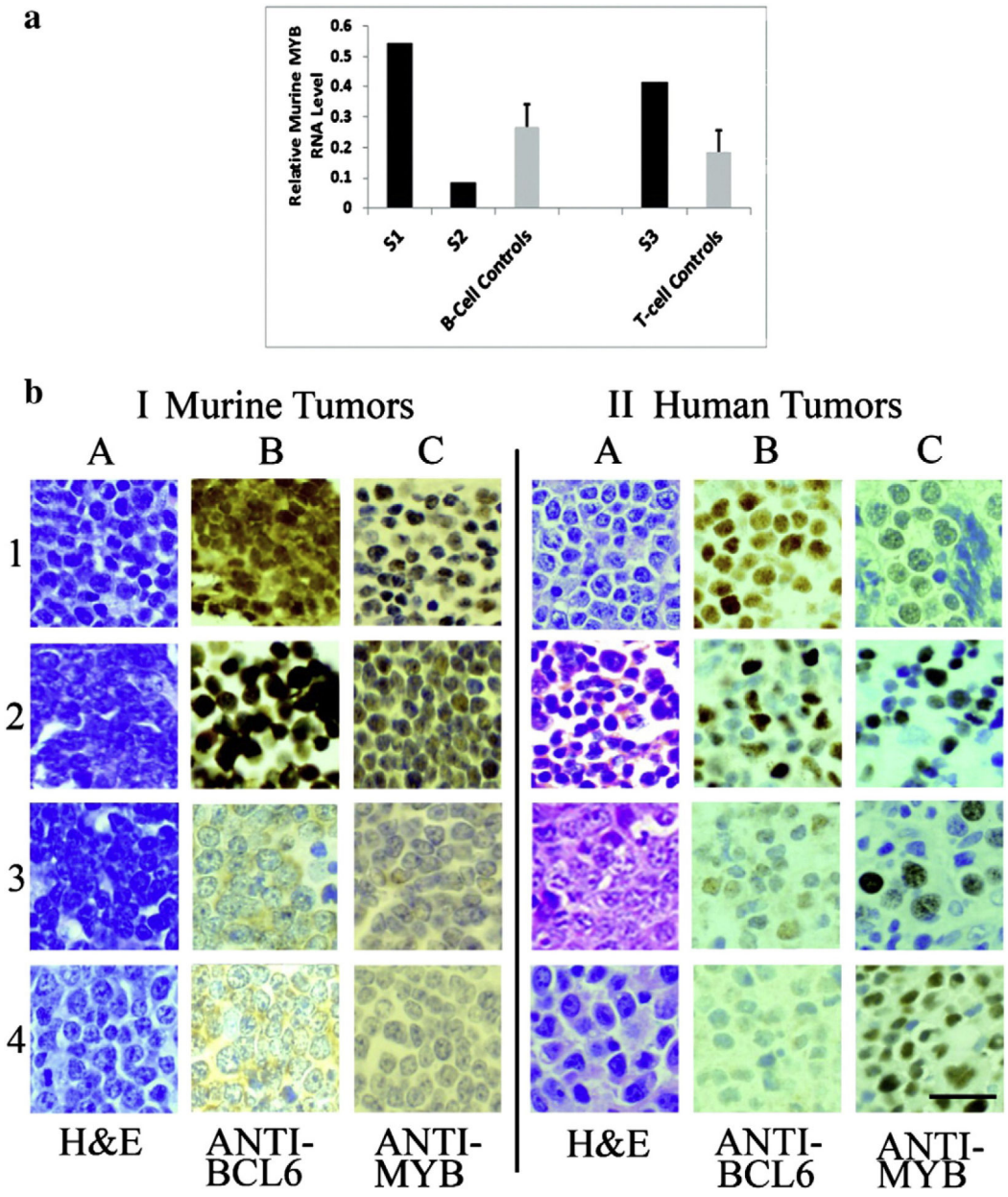

Fig. 3.

MYB: relative RNA levels in lymphomas from *BCL6* transgenic and control mice and immunohistochemistry of BCL6-positive and -negative murine and human lymphomas. (a) The graph depicts average relative MYB RNA expression in the lymphomas of three retroviral-injected *BCL6* transgenic mice (black bars, study mice S1, S2, S3) containing inserts ~35 to ~74 kb 3′ to the *MYB* gene as compared with randomly selected B- or T-cell lymphomas (gray bars) from retroviral-injected control mice that did not contain inserts in or near *MYB*. S1 is a large B-cell lymphoma from a transgenic mouse whose relative expression is 2-fold higher than the mean of three randomly selected B-cell control tumors (S1 expression = 0.54 vs. control B-cell tumors, mean ± SEM = 0.27 ± 0.07), whereas the relative RNA expression of S2 (0.08), also a large B-cell lymphoma from a transgenic

animal, is 3.2-fold decreased as compared with the mean of the three randomly selected control B-cell lymphomas (see above). S3 is a precursor T-cell lymphoblastic lymphoma/leukemia from a transgenic mouse whose relative RNA expression is 2.2-fold higher than the mean of two randomly selected precursor T-cell lymphoblastic lymphoma/leukemia control tumors; S3 expression = 0.42 vs. control T-cell tumors, mean ± SEM = 0.19 ± 0.07. (b) I, Representative murine lymphomas: tumors (1) and (2) depict the B- and T-cell lymphomas, respectively, from transgenic mice (BCL6-positive nuclear staining, column B) with positive *MYB* nuclear staining (column C); tumors (3) and (4) show B- and T-cell lymphomas, respectively, from non-transgenic mice (nuclei do not stain with anti-BCL6, column B); staining with anti-MYB is also negative in these animals (column C). (b) II, Representative human lymphomas: tumors (1) and (2) show BCL6-positive (column B) B- and T-cell lymphomas, respectively, which show positive nuclear staining for MYB (column C). Tumor (3) is a T-cell lymphoma that is weakly BCL-6 positive (column B), and tumor (4) is a BCL-negative T-cell neoplasm; in tumors 3 and 4, the cells that are MYB positive (column C) are BCL-negative. The bar (lowest right panel) depicts 50 μm.

Table 1

Location of retroviral inserts in transgenic mice and relative RNA expression levels in lymphomas from *BCL6* transgenic mice as compared with lymphomas from retroviral-injected non-transgenic (control) mice.

Mouse gene (chromosome)	Lymphoma type	Location of insert	Expression level vs. control
GFI1B (Chr 2)	B	8.692 kb 5′ to *GFI1B*	5.7-fold decreased
GFI1B	B	8.686 kb 5′ to *GFI1B*	2.6-fold decreased
GFI1B	B	7.877 kb 5′ to *GFI1B*	1.8-fold decreased
EVI5 (Chr 5)	T	Terminal intron*	2.0-fold decreased
EVI5	T	3′ untranslated region**	1.5-fold decreased
EVI5	T	3′ untranslated region***	1.3-fold decreased
MYB (Chr 10)	T	34.794 kb 3′ to *MYB*	2.2-fold increased
MYB	B	73.684 kb 3′ to *MYB*	2.0-fold increased
MYB	B	70.687 kb 3′ to *MYB*	3.2-fold decreased

* Nucleotide position 108,201,513.

** Nucleotide position 108,183,744.

*** Nucleotide position 108,182,569.

Table 2

Recurring retroviral integration sites in *BCL6* transgenic mice not found in non-transgenic controls.*

Murine genes containing inserts or, if insert not within a gene, the nearest characterized gene (unless indicated)	Mouse chromosome	Number of mice	Lymphoma type
PIM1 (proviral integration site for Moloney murine leukemia virus 1)	17	7	6T, 1B
GFI1B (growth factor independent 1B)	2	3	B
EVI5 (ecotropic viral integration site 5)	5	3	T
MYB (transcriptional activator MYB isoform 1)	10	3	2B, 1T
LMO2 (LIM domain only 2)	2	2	T
MN1 (probable tumor suppressor protein MN1)	5	2	T
AHI1 (jouberin isoform 1; Abelson helper integration site-1)	10	2	T
BCOR (BCL6 corepressor isoform d)	10	2	B
IKZF1 (DNA-binding protein Ikaros isoform a and b)	11	2	T
SYKb (tyrosine protein kinase SYK)	13	2**	T
MPPE (metallophosphoesterase 1); *IMPA2* (inositol monophosphatase 2)	18	2***	B
DNTT (DNA nucleotidylexotransferase)	19	2	T
GATA1 (erythroid transcription factor)	X	2	B

* Sequences have ≥98% identity to the murine databases described in the text.

** In one lymphoma, the insert is within *SYKb;* in the other, *SYKb* is 61.3 kb 3′ to the insert, but there is a gene closer to the insert at the 5′ side [40.3 kb away, *D/RAS2* (GTP-binding protein DI-RAS2)].

*** *MPPE* (5′) is closer to the insert in one lymphoma (17.8 kb vs. 50.2 kb), but *IMPA2* (3′) is closer to the insert in the other lymphoma (1 kb vs. 33.8 kb).