A Novel Recurrent Chromosomal Inversion Implicates the Homeobox Gene *Dlx5* in T-Cell Lymphomas from Lck-Akt2 Transgenic Mice

Yinfei Tan,[1] Roman A. Timakhov,[1,3] Mamta Rao,[1,4] Deborah A. Altomare,[1] Jinfei Xu,[1] Zemin Liu,[1] Qingshen Gao,[5] Suresh C. Jhanwar,[6] Antonio Di Cristofano,[1] David L. Wiest,[2] Janice E. Knepper,[1,4] and Joseph R. Testa[1]

[1]Human Genetics Program and [2]Immunobiology Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania; [3]Department of Molecular Biology, Russian State Medical University, Moscow, Russia; [4]Department of Biology, Villanova University, Villanova, Pennsylvania; [5]Department of Medicine, Northwestern University, Evanston, Illinois; and [6]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York

Abstract

The oncogene v-*akt* was isolated from a retrovirus that induced murine thymic lymphomas. Transgenic mice expressing a constitutively activated form of the cellular homologue *Akt2* specifically in immature T cells develop spontaneous thymic lymphomas. We hypothesized that tumors from these mice might exhibit oncogenic chromosomal rearrangements that cooperate with activated Akt2 in lymphomagenesis. Cytogenetic analysis revealed a recurrent clonal inversion of chromosome 6, inv(6), in thymic lymphomas from multiple transgenic founder lines, including one line in which 15 of 15 primary tumors exhibited this same rearrangement. Combined fluorescence *in situ* hybridization, PCR, and DNA sequence analyses showed that the distal inv(6) breakpoint resides at the T-cell receptor β chain locus, *Tcrb*. The proximal breakpoint maps to a region near a locus comprising the linked homeobox/transcription factor genes *Dlx5* and *Dlx6*. Expression analysis of genes translocated to the vicinity of the *Tcrb* enhancer revealed that *Dlx5* and *Dlx6* are overexpressed in tumors exhibiting the inv(6). Experimental overexpression of Dlx5 in mammalian cells resulted in enhanced cell proliferation and increased colony formation, and clonogenic assays revealed cooperativity when both Dlx5 and activated Akt2 were coexpressed. In addition, DLX5, but not DLX6, was found to be abundantly expressed in three of seven human T-cell lymphomas tested. These findings suggest that the *Dlx5* can act as an oncogene by cooperating with *Akt2* to promote lymphomagenesis. [Cancer Res 2008;68(5):1296–302]

Introduction

Clonal chromosome translocations and inversions are common in leukemias and lymphomas, and the characterization of such abnormalities has led to the identification of genes critical to the origin of these malignancies (1). In lymphoid malignancies, these rearrangements classically involve juxtaposition of a proto-oncogene, often encoding a transcription factor or signaling molecule (2), with a T-cell receptor (TCR) or immunoglobulin gene. As a result of these rearrangements, a proto-oncogene that is silent or expressed at low levels in T- or B-cell progenitor cells is activated when placed under the control of potent enhancer elements within the regulatory region of a TCR or immunoglobulin gene, respectively. In T-cell malignancies, activation of various transcription factor genes, including the *HOX11* homeobox gene, *MYC, LMO1/2, TAL1/2*, and *LYL1*, via juxtaposition to a TCR gene is common (2). Another example is the *TCL1* locus, which is activated in mature T-cell leukemias by translocations/inversions juxtaposing it to regulatory elements of TCR genes (3). Interestingly, functional analysis of TCL1 has revealed its involvement in the Akt prosurvival pathway by binding to Akt and enhancing its enzymatic activity (3).

Akt was first identified as an oncogene transduced by a murine retrovirus that induces thymic lymphomas (4), and Akt is frequently hyperactivated in human solid tumors and hematologic malignancies (5). Transgenic mouse models in which an Lck promoter was used to drive expression of constitutively activated forms of Akt1, Akt2, or Akt3 resulted in spontaneous T-cell lymphomas (6–8). The latent period for tumor development in these mice and the fact that not all T cells undergo transformation suggest that additional genetic events are required for full transformation. We hypothesized that genes that cooperate with *Akt* in lymphomagenesis might be identified due to their position near translocation junctions in tumors arising at the time when the T-lymphoid recombination system is active. Thus, to identify potentially cooperating oncogenic rearrangements in transgenic mice expressing activated Akt2 in the T-cell compartment, we performed cytogenetic and molecular genetic analyses on a series of lymphomas from these mice. A recurrent chromosomal rearrangement, involving the TCR β chain gene, *Tcrb*, and a locus comprising the homeobox/transcription factor genes *Dlx5* and *Dlx6*, is reported here.

Materials and Methods

Transgenic mice. Transgenic mouse lines were generated using a previously described construct (6, 8). Mice were genotyped by PCR using primers for HA and Akt2 (Table 1).

Cell lines and reagents. T cells were isolated from thymic lymphomas by passing tumor tissue through a 100 μ nylon mesh (BD Falcon) and culturing in Iscove's MDM containing 20% fetal bovine serum (FBS). ExactaCruz D immunoprecipitation kits and antibodies raised against Dss1 (N-15 and FL-70), Tcrb (H-197), Dlx5 (C20 and Y20), and Dlx6 (C20) were from Santa Cruz Biotechnology. Other anti-Dss1 antibodies were generated by one of us (Q.G.). A monoclonal Tcrb antibody (H57-597) was from BD PharMingen. Other antibodies included anti-HA (16B12) from Convance, anti–phospho-Akt from Santa Cruz or Cell Signaling Technologies, and anti–β-actin from Santa Cruz or Sigma. Functional studies of Dlx5 were performed with JML-5 cells (called Scid-Tacε cells in ref. 9), derived from a thymic lymphoma that arose spontaneously in a severe combined immunodeficient mouse, as well as with Rat-1 fibroblast cells.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Y.F. Tan and R.A. Timakhov contributed equally to this work.

Requests for reprints: Joseph R. Testa, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111. Phone: 215-728-2610; Fax: 215-214-1623; E-mail: Joseph.Testa@fccc.edu.

doi:10.1158/0008-5472.CAN-07-3218

Table 1. Primers and PCR conditions used in this investigation

Procedures	Primer name	Primer sequence	PCR condition
Genotyping of Lck-Akt2 mice	HA tag forward Akt2 reverse	AGGCACTGCCCTCTTGAAGC TTTGGGGTTCTGAATGTGAG	94°C for 5 min; 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and 72°C for 10 min
Cloning of inversion breakpoints	Dss1 forward TCRb reverse	TCAAAACTTTCGATCTGAATGGCTCC CATTTGGCCTCAATGTCCAGATGAGT	95°C for 5 min; 40 cycles of 95°C for 20 sec, 59°C for 20 sec, and 72°C for 1 min; and 72°C for 10 min
Multiplex RT-PCR	Dlx5 forward Dlx5 reverse Dlx6 forward Dlx6 reverse Ldb1 forward Ldb1 reverse	ACAACCGCGTCCCGAGTGCC CCCATCTAATAAAGCGTCCCGG CCAGGCTTTAAACCATCGCTTTC AATGCTGCCATGTTTGTGCAGATT GGAAGGCCGGTTGTACCTGGA CACCCCCCGAGCTCATGGTG	94°C for 4 min; 40 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min; and 72°C for 10 min

Karyotypic analysis and fluorescence *in situ* hybridization. Preparation of metaphase spreads and trypsin-Giemsa (G) banding were performed according to standard procedures. Chromosome identification and karyotype designations were in accordance with the University of Washington guidelines.[7]

Bacterial artificial chromosome (BAC) clones used for fluorescence *in situ* hybridization (FISH) were purchased from Children's Hospital Oakland Research Institute. BAC DNA was extracted using PSI clone BIG BAC DNA kit (Princeton Separations). For each probe, 500 ng of BAC DNA were labeled with either Spectrum Orange or Spectrum Green (Vysis) by random priming, using a BioPrime DNA Labeling kit (Invitrogen). Labeled probe was purified using sephadex G-50 columns (GE Healthcare). Probe was mixed with mouse Cot-1 DNA (Invitrogen) and precipitated with 3 mol/L sodium acetate and 100% ethanol. Precipitated probe was washed and resuspended in Hybrisol VII (MP Biomedicals) before hybridization. Smaller FISH probes were labeled by nick translation using DNA polymerase I/Dnase I (Invitrogen). Hybridization of probes to metaphase spreads and detection of FISH signals were performed according to standard procedures.

Cloning and sequencing of breakpoints. A PCR-based strategy was used to clone the genomic breakpoints of the inv(6), using primers near the breakpoints in *Tcrb* and *Dss1* (Table 1). PCR products were cloned using TOPO-TA (Invitrogen).

RNA extraction and Northern blotting. RNA was isolated using a Totally RNA kit (Ambion), following the manufacturer's recommendations. Northern blotting was performed using a Northern Max Ambion kit, per recommended procedures. The Dlx5 probe was a full-length cDNA, a gift from Giorgio Merlo (Dulbecco Telethon Institute, Milano, Italy). The Dss1 probe was a sequence corresponding to base numbers 1 to 114 of the coding region of *Dss1*. The β-actin probe provided with the Northern Max Ambion kit was used as an internal control. Probe (25 ng) was labeled with α-P^{32}-dCTP or γ-P^{32}-dCTP by random priming, using the Prime-It II Random Primer Labeling kit (Stratagene). Labeled probes were purified using a sephadex G-50 column, mixed with mouse Cot-1 DNA, placed in hybrisol solution, and hybridized to GeneScreen Plus membrane (PerkinElmer).

Reverse transcription-PCR. Semiquantitative multiplex reverse transcription-PCR (RT-PCR) was performed using SuperScript II reverse transcriptase (Invitrogen) with oligo dT. Multiplex RT-PCR was carried out using primers, and conditions were shown in Table 1. For real-time PCR, 100 or 20 ng of DNase-treated total RNA were used in reverse transcriptase reactions. Five microliters of cDNA were used in a 25-μL final volume. Primers for actin and Dlx5 were purchased from Applied Biosystems. The sequence of the Taq Man fluorogenic probe for the β-*actin* gene was 5′-CAGGAGTACATGAGTCCGGCCCC-3′, and the sequence for *Dlx5* was 5′-CCAGCCAGCCAGAGAAAGAAGTGGC-3′.

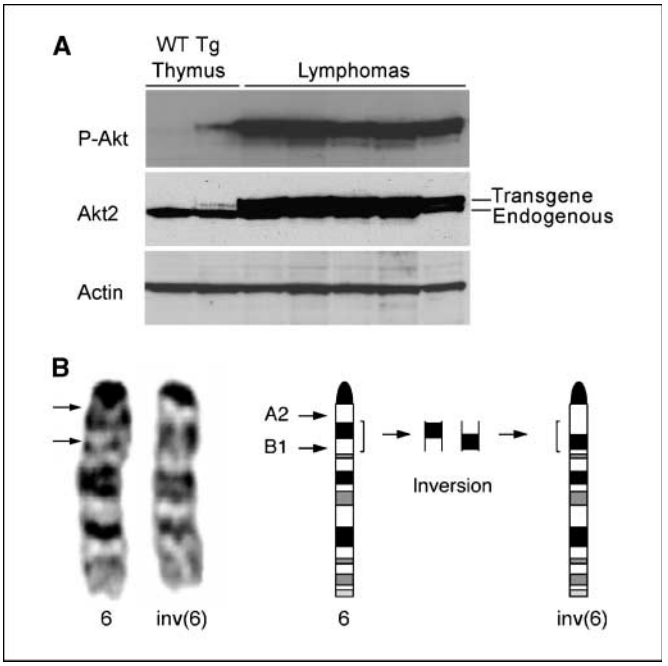

Figure 1. Akt activity and chromosome 6 inversion in Lck-Myr-Akt2 mice. *A,* Western blot analysis (*top*) demonstrating activated Akt in histologically normal thymus from 4-wk-old Lck-Myr-Akt2 mouse and increased levels of phospho-Akt in five inv(6)-positive thymic lymphomas. Immunoblotting was performed with a phospho-specific Akt antibody. *Middle,* immunoblotting with Akt2 antibody. *Bottom,* immunoblotting with β-actin antibody demonstrating even loading among lanes. *WT,* thymus from wild-type mouse; *Tg,* thymus from transgenic mouse. *B,* Giemsa banding of normal 6 and inv(6)(A2B1) from a thymic lymphoma of an Lck-Myr-Akt2 mouse (*left*). Note that pale band below the centromeric region is larger in the inv(6). Breakpoints involved in the inversion are depicted beside the normal chromosome 6. *Right,* low-resolution idiograms illustrating the chromosomal segment that becomes inverted in the inv(6). The inversion results in the juxtaposition of most of the pale G-bands 6A2 and 6B1.

[7] http://www.pathology.washington.edu/research/cytopages/idiograms/mouse/

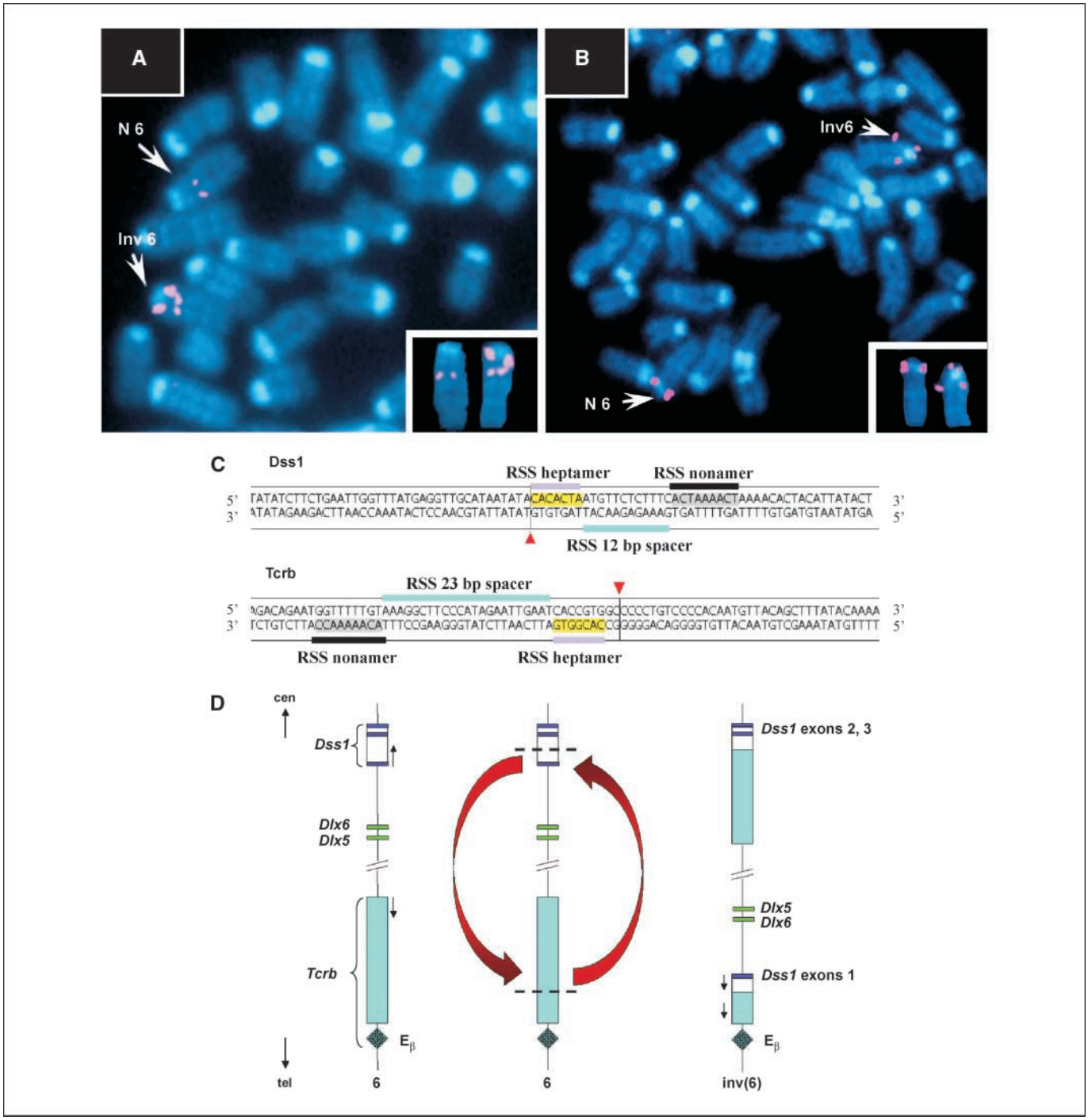

Figure 2. FISH and sequence analysis of breakpoints involved in the inv(6). *A,* FISH mapping of lymphoma with inv(6) showing that the *distal* breakpoint resides in *Tcrb* locus. BAC clone RP23-442M8 (*red*) localizes to 6B1 on normal 6 (*N6*) but is split in inv(6). BAC RP23-442M8 encompasses part of the variable and constant regions of *Tcrb*, including the enhancer. *B,* delineation of the *proximal* breakpoint of inv(6) from the same tumor. In normal 6, signals obtained with BAC clone RP24-204P9 map to the centromeric region. In inv(6), the signal is split. This BAC clone encompasses the entire *Dss1* locus. Insets in *A* and *B* align normal 6 (*left*) with inv(6) (*right*). *C,* DNA sequences demonstrating that breakpoints of inv(6) are located near the RSS site located in diversity gene D1 of the *Tcrb* locus and a cryptic RSS site in intron 1 of the *Dss1* gene. *Red arrowheads,* inv(6) breakpoint locations in relation to genomic sequences in the normal chromosome 6. Heptamer (*yellows*) and nonamer (*gray*) sequences similar to RSS are found adjacent to inv(6) breakpoints. *D,* schematic diagram of the inv(6). Inversion breakpoints (*dashed lines*) are in *Tcrb* locus and intron 1 of *Dss1*. The rearrangement places the *Tcrb* enhancer (*E*) near the *Dlx5* and *Dlx6* genes. Orientation is from centromere (*cen*) to telomere (*tel*).

Immunoprecipitation and Western blotting. Cells were incubated in lysis buffer on ice for 15 min. Protein quantitation was determined by the Bradford method. For immunoprecipitation, 1 mg protein lysates were incubated with immunoprecipitation matrix–Dlx6 antibody overnight at 4°C on a rotator. Washed pellets were resuspended in 2X reducing buffer and loaded onto a Novex SDS-PAGE gel (Invitrogen). For immunoblotting, samples (50 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblots were incubated with

primary antibodies at 4°C overnight, followed by incubation with secondary antibody conjugated with horseradish peroxidase for 60 mins at room temperature.

Retroviral transduction of lymphocytes. Phoenix-E retroviral packaging cells were transfected with murine stem cell virus–based pMiGII retroviral vectors using the calcium phosphate transfection method. Cells were maintained in complete Iscove's medium containing 20% FBS. JML-5 cells at a concentration of 1×10^6/mL were infected for 12 h at 37°C with retroviral supernatant. At 30 h postinfection, cells were isolated by flow cytometry for analysis. Transduction efficiencies were evaluated by determining the percentage of green fluorescent protein (GFP)-positive cells using flow cytometry. For the proliferation assay, GFP-positive cells were sorted, and then 10^4 cells per well were suspended in 100 μL of medium in 96-well plates. Cell numbers were determined at 24 h and 48 h using a hemacytometer.

Soft agar assay. Rat-1 fibroblasts (2×10^6 cells) were seeded in 6-well plates and incubated at 37°C overnight. Cells were cotransfected with 2 μg of each plasmid by using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the cells were selected by culturing in medium containing 100 μg/mL Hygromycin (Invitrogen) and 400 μg/mL G418 (Invitrogen). Two weeks after selection, cells were trypsinized and counted. Then, 1×10^4 cells from each stable cell line were suspended in 1 mL RPMI containing 10% FBS. One milliliter of 0.6% agar solution (diluted from fresh 3% Noble Agar with RPMI 1640 containing 10% FBS) were added to the cells. The mixture was poured into the 6-well plates prefilled with 2 mL of 0.6% bottom agar. The plates were left at room temperature for 20 min and then incubated at 37°C. Cells were fed weekly with 1 mL RPMI containing 10% FBS. After 14 days of incubation, cells were fixed with 10% formaldehyde in PBS for 5 min and stained with 0.5 mL of 0.005% Crystal Violet (Sigma) for 1 to 2 h. All colonies with >200 cells were counted.

Results

Five independently derived founder lines were generated using a previously described Lck-Myr-Akt2 vector (7) in which the Lck promoter is used to direct expression of myristylated, constitutively active Akt2 in immature T lymphocytes. Thymic lymphomas developed in 80% to 100% of animals from each line, with a mean latency of 11 to 19 weeks in different lines. Immunoblotting showed the presence of activated Akt in histologically normal thymus from 4-week-old transgenic mice, with increased levels in lymphomas (Fig. 1*A*).

Karyotyping revealed a novel chromosome 6 inversion, inv(6)(A2B1) (Fig. 1*B*), in lymphomas from three founder lines. FISH analysis using an Lck-Myr-Akt2 plasmid as probe revealed that the transgene integration site involved different chromosomes (3, 7, and 10) in the three founder lines exhibiting the inv(6). One founder line showed the inv(6) in all 15 lymphomas examined, whereas nonmalignant tissues from the same mice had normal karyotypes. To our knowledge, such consistency of a specific rearrangement in a transgenic founder line has not been previously described.

To map the inv(6) breakpoints, FISH was performed on tumor metaphases, using numerous BAC probes corresponding to known map locations. The distal breakpoint was mapped to the TCR β chain locus (*Tcrb*) in band 6B1, specifically near the enhancer (Fig. 2*A*). The proximal breakpoint was localized to an ~100-kb region encompassing the *Dss1* gene (Fig. 2*B*). Finer FISH mapping (data not shown), using long-range PCR-generated probes corresponding to 3′ and 5′ genomic sequences in *Dss1*, revealed that the proximal inv(6) breakpoint resides within this gene.

The proximal breakpoint junction was cloned using a PCR-based strategy, using primers corresponding to genomic sites within *Dss1* and *Tcrb*, close to each inversion breakpoint (Supplementary Fig. S1). All primers were designed such that each would be in the sense orientation in the normal chromosome 6 and, therefore, would not result in a PCR product. However, in the inv(6), some primers would be oriented in the antisense direction and near enough to result in a PCR product. Using this strategy, a ~4.5-kb PCR product was obtained in genomic DNA from 4 of 10 inv(6)-positive lymphomas tested. PCR products from two tumors were cloned and sequenced to confirm disruption of *Dss1*. Sequence analysis of the breakpoints revealed the presence of a consensus recombination signal sequence (RSS) site at the breakpoint in *Tcrb* and a variant RSS in *Dss1* (Fig. 2*C*), suggesting RAG-mediated variable, diversity, and joining [V(D)J] recombination. A schematic diagram of the inv(6) is shown in Fig. 2*D*.

Northern blot analysis showed that inv(6) lymphomas exhibit an endogenous *Dss1* transcript and a larger transcript, representing a *Dss1* exon1-*Tcrb* fusion transcript (Supplementary Fig. S2). This was confirmed by RT-PCR analysis in 20 of 20 inv(6)-positive tumors tested, and sequencing of the PCR product revealed a chimeric cDNA fragment consisting of *Dss1* exon1 fused in-frame

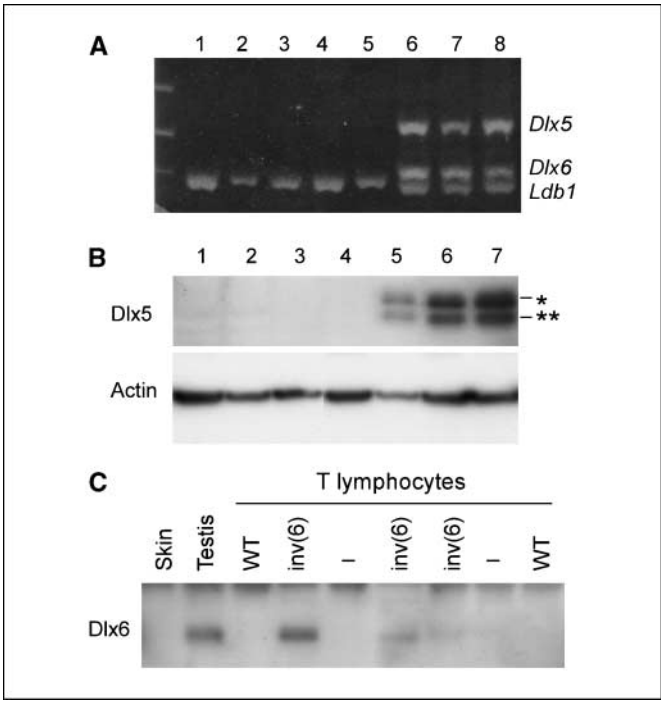

Figure 3. Deregulated expression of Dlx5 and Dlx6 in inv(6) lymphomas. *A,* multiplex RT-PCR analysis of expression of *Dlx5* and *Dlx6*. *Lanes 1* to *4,* Lck-Myr-Akt2 lymphomas without inv(6); *lane 5,* thymocytes from a normal wild-type mouse; *lanes 6* to *8,* Lck-Myr-Akt2 lymphomas with inv(6). *Ldb1* (Lim domain binding 1) is an internal loading control. *B,* Western blot analysis demonstrating consistently robust up-regulation of Dlx5 protein in inv(6)-positive lymphomas from Lck-Myr-Akt2 mice. *Lanes 1* to *3,* lymphomas from tumors without the inv(6); *lane 4,* wild-type murine thymocytes; *lanes 5* to *7,* lymphomas with inv(6). *, top band is 38 kDa, which corresponds to the size of Dlx5 protein reported elsewhere (19, 20); **, bottom band is ~34 kDa and may represent one of several predicted alternative splice isoforms of Dlx5 (19, 20). *C,* immunoprecipitation/Western showing up-regulation of Dlx6 protein in inv(6) lymphomas. Variable Dlx6 expression is seen in three inv(6) lymphomas, whereas Dlx6 was not detected in lymphomas without the inversion (–) or in thymocytes from wild-type mice. Samples were homogenized using radioimmunoprecipitation assay buffer; then 1-mg protein lysate from tissue or T cells was immunoprecipitated with Dlx6 C20 antibody (Santa Cruz), followed by immunoblotting using Dlx6 C20 antibody. Skin and testis are negative and positive controls, respectively.

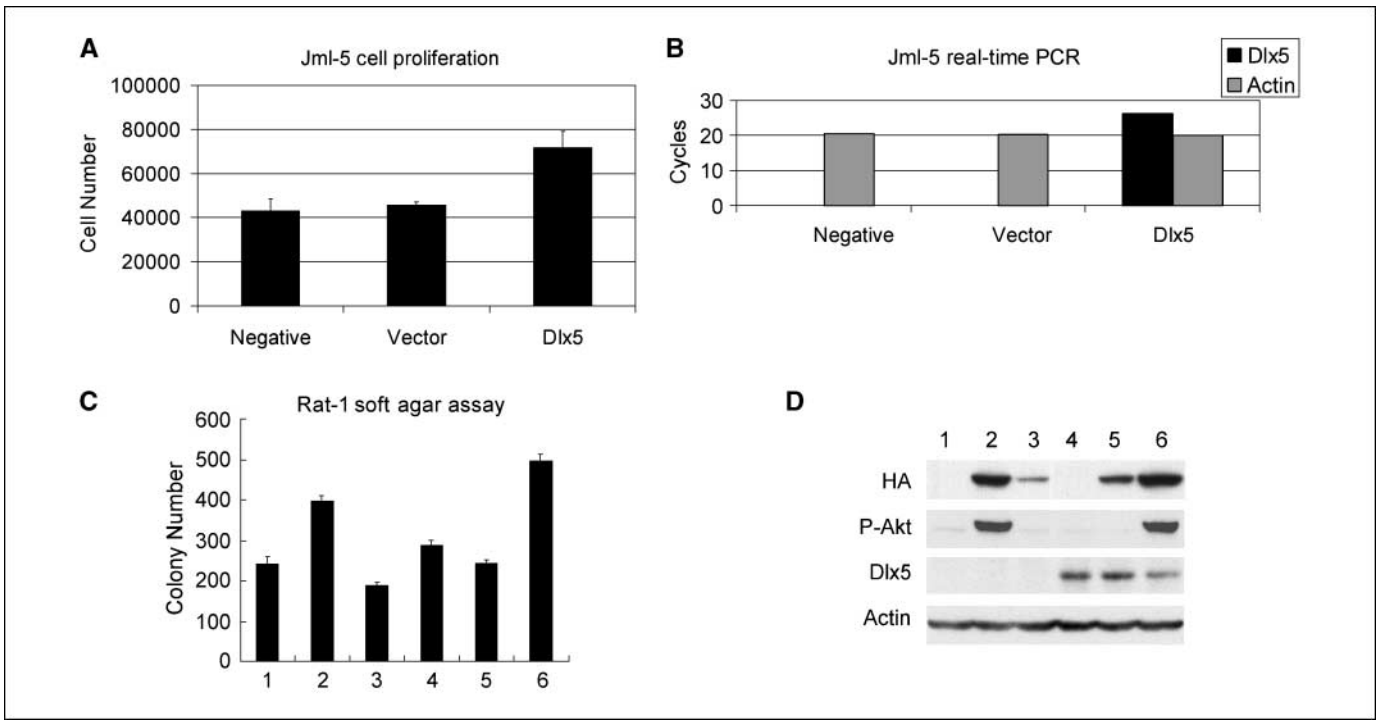

Figure 4. Expression of Dlx5 contributes to increased cell proliferation and growth in soft agar. *A,* transduction of JML-5 cells with Dlx5 accelerates cell proliferation. Control or retrovirally transduced GFP-positive JML-5 cells were isolated by flow sorting, and then 10^4 cells per well were seeded into 100 μL of medium. Cell numbers were determined after incubating for 24 h. Figure depicts one of three replicate experiments. *Columns,* mean; *bars,* SD. *B,* graphical representation of the number of cycles needed to detect control *actin* or *Dlx5* expression in untransduced (negative) cells, cells transduced with vector (vector), and cells transduced with *Dlx5* (Dlx5) for 24 h. Note that *Dlx5* expression was not detectable in untransduced or vector-transduced JML-5 cells. *C,* overexpression of Dlx5 enhances colony formation in soft agar. Rat-1 cells were cotransfected and selected by Hygromycin and G418 for 2 wk. Cells were plated in soft agar, and colony numbers were counted after 2 wk of growth. The plasmid combinations were as follows: 1, pcDNA3.1-Hygro (*vec1*) + pcDNA3.0-Neo (*vec2*); 2, pcDNA3.1 + pcDNA3.0-HA-MyrAkt2; 3, pcDNA3.1 + pcDNA3.0-HA-Akt2E299K; 4, pcDNA3.1-Dlx5 + pcDNA3.0; 5, pcDNA3.1-Dlx5 + pcDNA3.0-HA-Akt2E299K; and 6, pcDNA3.1-Dlx5 + pcDNA3.0-HA-MyrAkt2. *D,* cell lysates were prepared from different stable cell lines. Western blots were screened with antibodies against HA epitope, phospho-Akt, Dlx5, and control β-actin.

to the constant region of *Tcrb*. *Dss1* exons2/3 were fused to the *Tcrb* D1 segment in an arrangement that precludes the potential for mRNA or protein production based on genetic database predictions. Quantitative RT-PCR showed that exon 1 was up-regulated, whereas exons 2 and 3 were not. However, Western blot analysis with anti-Dss1 antibodies raised against either the full-length protein or amino terminus did not detect a Dss1-Tcrb fusion protein in inv(6) lymphomas. Similarly, immunoblot analysis with Tcrb antibodies, and immunoprecipitation/Westerns using various combinations of Dss1 and Tcrb antibodies, did not detect a fusion protein. Sequencing of the remaining *Dss1* allele from six different inv(6) lymphomas did not reveal any point mutations.

Because oncogenic rearrangements involving TCR loci classically result in translocation of an intact gene into an abnormal regulatory context, the genomic region juxtaposed with the *Tcrb* enhancer was therefore explored for intact candidate genes that might be affected. Database searches revealed two genes (*Dlx5* and *Dlx6*) within 250 to 300 kb of the *Tcrb* enhancer in inv(6) lymphomas. To determine if expression of *Dlx5* and/or *Dlx6* was up-regulated in inv(6) lymphomas, we used multiplex RT-PCR (Fig. 3*A*) and verified the findings by real-time PCR (data not shown). Northern blot analysis also confirmed up-regulation of *Dlx5* (Supplementary Fig. S2). Although greatly elevated expression of both *Dlx* genes was observed in inv(6) lymphomas, transcripts were not detected in thymocytes from wild-type mice or other T-cell lymphoma cell lines without the inv(6). Immunoblot analysis documented consistent, greatly up-regulated expression of Dlx5 in inv(6) lymphomas and no detectable Dlx5 in normal thymocytes or lymphomas without the inv(6) (Fig. 3*B*). In our hands, commercially available anti-Dlx6 antibodies have not worked for direct Westerns, although we detected varying levels of up-regulation of Dlx6 protein in inv(6) lymphomas by immunoprecipitation/Western analysis (Fig. 3*C*). We also used real-time RT-PCR to examine expression of *Evf1/2* and several imprinted genes that cluster in the *Dlx5/Dlx6* region of chromosome 6 (Supplementary Fig. S3). Expression of a *Dlx6*

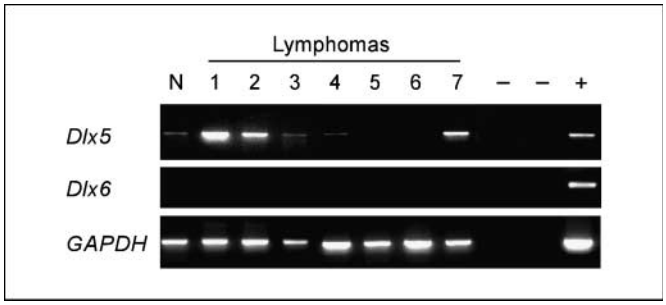

Figure 5. RT-PCR analysis depicting expression of *DLX5* and *DLX6* in seven human T-cell lymphomas compared with expression in a nonmalignant lymph node (*N*). Up-regulation of *DLX5* was observed in three lymphomas, whereas none of the clinical tumor specimens showed up-regulation of *DLX6*. (–), negative control; (+), positive control. *GAPDH* was used as a loading control.

antisense RNA, *Dlx6-as*, was examined by semiquantitative RT-PCR. None of the imprinted genes translocated near the proximal inv(6) breakpoint were up-regulated in inv(6) lymphomas compared with normal thymocytes. Instead, up-regulation was limited to *Dlx5, Dlx6, Evf1/2*, and *Dlx6-as*, each of which is located near the distal inv(6) breakpoint, driven by the 3′ enhancer of *Tcrb*. Expression of *Acn9*, located ~375 kb distal to *Dss1*, was not elevated in inv(6) lymphomas.

To determine whether Dlx5 can enhance cell proliferation, we used a retroviral vector to express Dlx5 in JML-5 cells. Overexpression of Dlx5 resulted in a 40% increase in cell number 24 h after plating (Fig. 4*A* and *B*).

Soft agar experiments indicated that exogenous overexpression of Dlx5 can promote anchorage-independent growth of Rat-1 fibroblasts, with about a 25% increase in the number of colonies formed by cells transfected with a plasmid expressing Dlx5 compared with that for cells transfected with an empty vector (Fig. 4*C* and *D*). These data provide support for our hypothesis that aberrant expression of *Dlx5* is oncogenic. Furthermore, coexpression of Dlx5 and Myr-Akt2 in Rat-1 cells resulted in a 25% increase in the number of colonies compared with Rat-1 cells expressing Myr-Akt2 alone (Fig. 4*C* and *D*), suggesting that *Dlx5* can cooperate with *Akt2* to promote clonogenic growth in soft agar. As expected, expression of dominant-negative Akt2 (E299K) decreased the number of colonies observed. In preliminary experiments, we were able to achieve significant knockdown of *Dlx5* with one shRNA, which resulted in decreased cell proliferation and decreased expression of cyclin D1 (Supplementary Fig. S4).

Finally, of potential clinical significance, *DLX5*, but not *DLX6*, was abundantly expressed in several human T-cell lymphomas (Fig. 5). Semiquantitative RT-PCR analysis revealed up-regulated *DLX5* transcript levels in three of seven patient-derived T-cell lymphomas compared with that observed in nonmalignant lymph node samples. Expression of *DLX6* was not detected in any of the human T-cell lymphomas or noncancerous lymph nodes tested.

Discussion

V(D)J recombination puts early thymocytes at risk for malignant transformation should the DNA breaks be rejoined inappropriately and cause the kind of chromosomal rearrangements associated with certain leukemias and lymphomas (2). For V(D)J recombination at the *Tcrb* locus, the fidelity of the rearrangement events is monitored by the β selection checkpoint, at which cells with aberrant *Tcrb* rearrangements are eliminated by apoptosis in the thymus (10). However, the concomitant expression of activated Akt has been shown to influence thymocyte selection and promote peripheral T-cell survival (11). Our working model of lymphomagenesis in Lck-Myr-Akt2 mice is that activation of the Akt pathway would prevent apoptosis in a T-cell progenitor that undergoes aberrant V(D)J recombination connected with the inv(6), whereas genes affected by the inversion would promote malignant transformation.

In T-lymphoid malignancies, tumor-specific translocations and inversions classically juxtapose potent enhancer elements of a TCR gene with a proto-oncogene often encoding a transcription factor such as *Myc* and *Hox11* (2). As a result, proto-oncogenes that are silent or expressed at low levels in T-lymphoid progenitor cells become activated (2).

In inv(6) lymphomas, the rearrangement places the *Tcrb* enhancer in the vicinity of two homeobox genes, *Dlx5* and *Dlx6*, resulting in up-regulated expression. *Dlx5* and *Dlx6* belong to a conserved superfamily of developmental regulatory genes encoding transcription factors involved in cell fate and tissue identity. Although not extensively studied in lymphoid progenitor cells, dysregulation of homeobox genes is increasingly recognized as contributing to hematologic malignancies. Expression of tightly linked *Dlx* genes, such as the *Dlx5* and *Dlx6* bigene pair, is often overlapping and partially redundant in function; and linked *Dlx* genes share *cis*-regulating sequences, i.e., intergenic regions containing enhancer elements (12). In mice, *Dlx5* is expressed at very low levels in whole thymus and is not expressed in Thy1+ thymocytes; *Dlx6* is not detected in the thymus (13). Interestingly, microarray studies have revealed up-regulation of *DLX5* in several human cancers, including endometrial carcinomas and lung carcinomas (14, 15), suggesting that overexpression of DLX5 could be oncogenic. Further evidence suggesting an oncogenic role of *DLX5* comes from our experiments illustrating that overexpression of Dlx5 in mammalian cells results in enhanced cell proliferation and increased colony formation, and that knockdown of *Dlx5* in inv(6)-positive thymic lymphoma cells may result in decreased cell proliferation. Moreover, we have knocked down the expression of *DLX5* in human ovarian cancer cells overexpressing *DLX5,* using several different shRNA constructs, and decreased expression of DLX5 coincided with a decrease in cyclin D1 expression and reduced cell proliferation.[8]

Recent work has shown constitutive activation of AKT in the majority of primary human lymphomas, supporting its proposed key role in lymphoma cell survival (16). Among the members of the AKT family, AKT2 may have particular importance in mediating PI3K-dependent oncogenic effects (17). Our work indicates that Akt2 activation plays an important role in the development of thymic lymphomas seen in a transgenic mouse model. However, the frequent occurrence of the inv(6) seen in tumors from these mice indicates that, in addition to *Akt2*, other genetic lesions are necessary for malignant transformation.

Collectively, our findings suggest that Dlx5 acts oncogenically by cooperating with Akt2 to promote T-cell lymphomagenesis. In addition to contributing to our understanding of the pathogenesis of lymphoma in this mouse model system, aberrant expression of DLX5 is also involved in some human malignancies (14, 15). Given the fact that DLX5 is up-regulated in some human endometrial and lung carcinomas, DLX5 could potentially be exploited therapeutically. AKT is often activated in human cancers, including lung and endometrial carcinomas (5). To date, use of AKT as a therapeutic target has been limited by toxicity (18), due to the fact that AKT signaling is ubiquitously involved in cell survival not only in transformed cells but also in normal tissue, and AKT2 plays a critical role in glucose metabolism (18). This problem might be mitigated, should DLX5 represent a "druggable" target that displays a more restricted expression pattern.

Acknowledgments

Received 8/20/2007; revised 11/26/2007; accepted 12/19/2007.

Grant support: National Cancer Institute Grants CA77429 and CA06927 and an appropriation from the Commonwealth of Pennsylvania.

[8] Y. Tan et al., in preparation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The authors thank Dr. Binaifer Balsara (Fox Chase Cancer Center) for technical assistance with some of the FISH experiments, Dr. Dietmar Kappes (Fox Chase Cancer Center) for expert scientific advice, and Dr. Philip Tsichlis (Tufts University, Boston) for providing one of the transgenic founder lines. The following Fox Chase Cancer Center shared facilities were used in the course of this work: Laboratory Animal, Genomics, Transgenic Mouse, Biochemistry and Biotechnology, Cell Culture, and Histopathology.

References

1. Rowley JD. Chromosome translocations: dangerous liaisons revisited. Nat Rev Cancer 2001;1:245–50.

2. Look AT. Oncogenic transcription factors in the human acute leukemias. Science 1997;278:1059–64.

3. Pekarsky Y, Hallas C, Croce CM. Molecular basis of mature T-cell leukemia. JAMA 2001;286:2308–14.

4. Staal SP, Hartley JW. Thymic lymphoma induction by the AKT8 murine retrovirus. J Exp Med 1988;167:1259–64.

5. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene 2005;24: 7455–64.

6. Malstrom S, Tili E, Kappes D, Ceci JD, Tsichlis PN. Tumor induction by an Lck-MyrAkt transgene is delayed by mechanisms controlling the size of the thymus. Proc Natl Acad Sci U S A 2001;98:14967–72.

7. Mende I, Malstrom S, Tsichlis PN, Vogt PK, Aoki M. Oncogenic transformation induced by membrane-targeted Akt2 and Akt3. Oncogene 2001;20:4419–23.

8. Rathmell JC, Elstrom RL, Cinalli RM, Thompson CB. Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. Eur J Immunol 2003;33: 2223–32.

9. Carleton M, Ruetsch NR, Berger MA, Rhodes M, Kaptik S, Wiest DL. Signals transduced by CD3ε, but not by surface pre-TCR complexes, are able to induce maturation of an early thymic lymphoma *in vitro*. J Immunol 1999;163:2576–85.

10. Petrie HT, Livak F, Burtrum D, Mazel S. T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production. J Exp Med 1995; 182:121–7.

11. Na SY, Patra A, Scheuring Y, et al. Constitutively active protein kinase B enhances Lck and Erk activities and influences thymocyte selection and activation. J Immunol 2003;171:1285–96.

12. Morasso MI, Radoja N. Dlx genes, p63, and ectodermal dysplasias. Birth Defects Res Part C Embryo Today 2005;75:163–71.

13. Woodside KJ, Shen H, Muntzel C, Daller JA, Sommers CL, Love PE. Expression of Dlx and Lhx family homeobox genes in fetal thymus and thymocytes. Gene Expr Patterns 2004;4:315–20.

14. Pedersen N, Mortensen S, Sorensen SB, et al. Transcriptional gene expression profiling of small cell lung cancer cells. Cancer Res 2003;63:1943–53.

15. Maxwell GL, Chandramouli GV, Dainty L, et al. Microarray analysis of endometrial carcinomas and mixed mullerian tumors reveals distinct gene expression profiles associated with different histologic types of uterine cancer. Clin Cancer Res 2005;11: 4056–66.

16. Fillmore GC, Wang Q, Carey MJ, Kim CH, Elenitoba-Johnson KS, Lim MS. Expression of Akt (protein kinase B) and its isoforms in malignant lymphomas. Leuk Lymphoma 2005;46:1765–73.

17. Arboleda MJ, Lyons JF, Kabbinavar FF, et al. Overexpression of AKT2/protein kinase Bβ leads to up-regulation of β1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res 2003;63:196–206.

18. Hanada M, Feng J, Hemmings, BA. Structure, regulation and function of PKB/AKT - a major therapeutic target. Biochim Biophys Acta 2004;1697: 3–16.

19. Kuwajima T, Nishimura I, Yoshikawa K. Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins. J Neurosci 2006; 26:5383–92.

20. Young JI, Hong EP, Castle JC, et al. Regulation of RNA splicing by the methylation-dependent transcriptional repressor methyl-CpG binding protein 2. Proc Natl Acad Sci U S A 2005;102:17551–8.

A Novel Recurrent Chromosomal Inversion Implicates the Homeobox Gene *Dlx5* in T-Cell Lymphomas from Lck-Akt2 Transgenic Mice

Yinfei Tan, Roman A. Timakhov, Mamta Rao, et al.

Cancer Res 2008;68:1296-1302.