13q14 deletions in CLL involve cooperating tumor suppressors

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B-cell chronic lymphocytic leukemia (CLL) is the most common human leuke-

mia. 13q14 deletions are most common chromosomal alterations in CLL. We previously reported that miR-15/16 is a target of 13q14 deletions and plays a tumor suppressor role by targeting BCL2. Because DLEU7 is located near miR-15/16 and is also positioned within a minimal deleted region, we investigated whether DLEU7 could also play a tumor suppressor role. Recent studies of transgenic mouse models demonstrated the importance of the nuclear factor-κB (NF-κB) pathway in CLL. To examine the possible role of DLEU7 in CLL, we investigated the effect of DLEU7 expression on NF-κB and nuclear factor of activated T cells (NFAT) activity. We found that DLEU7 functions as a potent NF-κB and NFAT inhibitor by physically interacting and inhibiting TACI and BCMA, members of the tumor necrosis factor (TNF) receptor family involved in B-CLL. In addition, DLEU7 expression in A549 lung cancer cells resulted in a decrease in S phase and increased apoptosis. The results suggest that loss of DLEU7 may cooperate with the loss of miR-15/16 in the pathogenesis of CLL. (Blood. 2010;115(19):3916-3922)

Introduction

Chronic lymphocytic leukemia (CLL) lymphocytes have mature appearance and the B220+CD5+ phenotype.1,2 Several chromosomal aberrations occur frequently in CLL cases, including 13q deletions (~50%), 11q23 deletions (18%), trisomy 12 (12%), and 17p deletions (7%).3 The 13q14 deletion is the most common B-CLL aberration and is associated with low levels of ZAP70 expression and mutated variable region genes of immunoglobulins.4 Analysis of an approximately 30-kb deletion at 13q14.3 and chromosomal breakpoint mapping of translocation t(2;13)(q32;q14) led to the discovery of 2 physically linked microRNAs, miR-15a and miR-16-1, as targets of these deletions.5 Furthermore, both, miR-15a and miR-16-1 were reduced in expression in most CLL cases,5 and further studies indicated that miR-15a/miR-16-1 negatively regulate Bcl2 expression.6 These findings indicated that down-regulation of miR-15/16 and subsequent Bcl2 up-regulation contribute to CLL pathogenesis.5 A high-resolution map of 13q14 deletions using 171 CLL samples was recently reported.7 These data indicated that the minimal deleted region, in addition to miR-15/16, also contains the DLEU7 gene (Figure 1A). DLEU7 was previously identified as a candidate tumor suppressor gene at 13q14.8 It encodes a 221–amino acid protein with no homology to known proteins. No mutations in DLEU7 were found in 45 CLL samples, although the DLEU7 promoter was methylation in 61% of CLL.8 Since DLEU7 is the only protein-coding gene located within the minimal deleted region at 13q14, we investigated whether DLEU7 can function as a tumor suppressor.

Methods

CLL samples and real-time PCR experiments

A total of 25 CLL samples were obtained after informed consent from patients diagnosed with CLL from the CLL Research Consortium. Research was performed with the approval of the Institutional Review Board of Ohio State University. Briefly, blood was obtained from patients with CLL, and lymphocytes were isolated through Ficoll/Hypaque gradient centrifugation (Amersham Biosciences) and processed for RNA extraction by using the standard TRIZol method (Invitrogen). Real-time polymerase chain reaction (PCR) experiments were carried out using Hs00415570_m1 (DLEU7), 000389 (miR-15a), and 000391 (miR-16-1) assays for real-time PCR (Applied Biosystems) according to the manufacturer’s protocol. GAPDH was used as a control.

DNA constructs

Expression constructs, containing full-length human DLEU7, TNFRSF13B (TACI), TNFRSF17 (BCMA), TNFRSF5 (CD40), and TRAF6 were purchased from OriGene. Expression constructs, containing Myc-tagged open reading frames (ORFs) of human TACI and BCMA, named TACI-myc and BCMA-myc, respectively, were also purchased from OriGene. Full-length human DLEU7 ORF with an HA tag was cloned into a pCMV5 vector to obtain the pCMV5-hDLEU7-HA construct. The GLTX5-HA construct was created by cloning GLTX5 ORF into the pCMV-HA vector (BD Biosciences). To obtain expression constructs encoding GFP-hDLEU7 and FHIT-GFP fusion proteins hDLEU7 and FHIT ORFs were cloned into a pEGFP-N1 vector from Clontech.

Dual-luciferase Reporter Assay System and Renilla luciferase reporter vector pRL-TK were purchased from Promega. The NF-κB reporter construct pNF-κB-Luc was purchased from Stratagene. The NFAT reporter
Figure 1. DLEU7 at 13q14. (A) Schematic representation of minimal deleted region at 13q14 in CLL (adapted from Ouillette et al7Fig2). (B) Expression of DLEU7, miR-15a, and miR-16-1 in B-CLL samples. Relative expression levels of DLEU7, miR-15a, and miR-16-1 in 25 B-CLL samples, 4 lymphoid cell lines, and in normal CD19+ cells were assayed by real-time PCR. Expression levels of DLEU7, miR-15a, and miR-16-1 in CD19+ cells was set at 1. Expression levels of DLEU7, miR-15a, and miR-16-1 were normalized to the expression of GAPDH.

construct pNFAT-Luc was purchased from Affymetrix. The construct encoding NFATC1 (NFAT2) under the cytomegalovirus (CMV) promoter, pCMV-NFAT, was purchased from OriGene.

Cell culture, transfection, Western blotting, and immunoprecipitation experiments

HEK 293, NIH-3T3, A549, K562, SUPT11, CA46 and Daudi cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 25 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO2 in air. Human CD19+ B cells were purchased from Lonza. FuGene 6 transfection reagent and protease inhibitor cocktail tablets were obtained from Roche. Transfections, except luciferase assay experiments; cell lysate preparations and Western blot analysis were carried out as described previously.10 Immunoblots were developed using Pierce ECL Western Blotting substrate or SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific. Human recombinant TNF-α was purchased from Calbiochem. Antibodies used for Western
6blots and immunoprecipitation experiments were: anti-Dleu7, anti-Myc, anti-Myc–HRP, anti-GFP, anti-GFP–HRP (Santa Cruz Biotechnology), anti-HA (Covance), and anti-HA–HRP (Roche).

**Immunofluorescence**

NIH-3T3 cells were grown on Human Fibronectin Cellware 2-Well CultureSlides (BD Biosciences). Immunofluorescence experiments were carried out as previously described using a Zeiss LCM 510 confocal microscope, a 63×/1.2 W water objective, and MBF ImageJ software. Secondary antibodies used for immunofluorescence were as follows: goat anti–mouse Alexa Fluor 546 (red) and goat anti–rabbit Alexa Fluor 488 (green), all purchased from Invitrogen.

**Cell-cycle and apoptosis analysis**

Recombinant adenovirus expressing human DLEU7 and GFP (Ad5-DLEU7) was obtained using the BD Adeno-X Expression System 1 (BD Biosciences) according to the manufacturer’s protocol. Packaged recombinant adenovirus particles were harvested by lysing transfected HEK 293 cells. Consequent cell-cycle and apoptosis experiments were performed in the A549 lung cancer cell line, which was infected with Ad5-control or Ad5-DLEU7 virus. Flow cytometry experiments were carried out on BD LSR II flow cytometer, controlled by BD FACSDiva software (BD Biosciences). Cell-cycle experiments were carried out using the Click-It EdU Flow Cytometry Assay Kit from Invitrogen according to the manufacturer’s protocol. Apoptotic cells were detected using APC annexin V from BD Biosciences and the MitoProbe DiIC1 (5) assay kit from Invitrogen.

**Luciferase assay**

HEK 293 cells were transfected with the indicated constructs. Firefly and renilla luciferase activities were assayed with the dual luciferase assay system (Promega) and firefly luciferase activity was normalized to renilla luciferase activities were assayed with the dual luciferase assay from Molecular Probes (Invitrogen).

**Results**

We first examined the expression of DLEU7, miR-15a and miR-16-1 in B-CLL samples. Figure 1B shows real-time reverse transcriptase (RT)–PCR results in normal CD19+ B cells, 4 leukemia cell lines (K562, SUPT11, CA46, and Daudi) and 25 CLL samples (1-25). All 25 B-CLL samples showed decreased DLEU7 expression, while 13 of 25 samples showed decreases of 10-fold or more (Figure 1B). miR-15a expression was decreased in all B-CLL samples compared with normal CD19+ B cells, and 23 of 25 samples showed decreases of 2-fold or more. Similarly, miR-16-1 expression was decreased in 24 of 25 samples (Figure 1B). These results are consistent with previously reported observations that DLEU7 essentially is not expressed in CLL, and that miR-15/16 expression is down-regulated in CLL.

We proceeded to determine whether DLEU7 could play a tumor suppressor role in CLL. Recent studies of transgenic mouse models demonstrated the importance of the NF-κB pathway in CLL (reviewed in Pekarsky et al). For example, transgenic expression of a proliferation-inducing TNF ligand (APRIL), a member of the tumor necrosis factor (TNF) superfamily involved in NF-κB activation, resulted in significant expansions of B220+CD5+ cells. Recently, we reported that Tc11 activation caused B-CLL by activation of the NF-κB pathway and inhibition of AP-1. Since recent functional studies and studies of animal models suggested a role for the NF-κB pathway in the pathogenesis of CLL, we examined the possibility that Dleu7 might function as an inhibitor of NF-κB. NF-κB can be routinely activated in mammalian cells by stimuli involving the tumor necrosis factor (TNF) receptor (TNF-R). In this experiment, we used a commercial NF-κB reporter construct, pNF-κB-Luc, expressing luciferase under the control of an NF-κB–responsive element. NIH-3T3 cells were transfected with the constructs indicated in Figure 2A, and the NF-κB reporter was activated by treatment with TNF-α for 2, 4, or 6 hours. Figure 2A shows that Dleu7 expression inhibits NF-κB activity approximately 2.5- to 3-fold in each of 3 time points. NF-κB activity could also be activated by overexpression of various molecules involved in TNF–NF-κB pathway. To confirm that Dleu7 can function as an inhibitor of NF-κB, we activated NF-κB activity in 293 cells by TRAF5, TRAF6, and CD40 and investigated whether Dleu7 inhibits NF-κB in these settings. HEK 293 cells were transfected with the constructs indicated in Figure 2B. Figure 2B shows that Dleu7 significantly inhibits NF-κB.

![Figure 2. Dleu7 inhibits NF-κB-dependent transcription.](https://www.bloodjournal.org) (A) NIH-3T3 cells were cotransfected with 250 ng of pNF-κB-Luc reporter and 50 ng of pRL-TK Renilla reporter constructs. In addition, 1.5 mg of CMV5-empty vector or 1.5 mg of CMV5-DLEU7-HA was used. In addition, cells were treated with 50 ng/mL TNF-α for 6, 4, or 2 hours before lysis where indicated. Firefly and Renilla luciferase activities were assayed with the Dual-Luciferase Assay System from Promega, and firefly luciferase activity was normalized to Renilla luciferase activity. The normalized promoter activity of pNF-κB-Luc in NIH-3T3 cells transfected with CMV5-empty vector without TNF-α treatment was set as 1. Experiments were repeated 3 times in triplicate. Columns indicate the mean; bars, SD. (B) HEK 293 cells were cotransfected with 50 ng of pNF-κB-Luc reporter and 50 ng of pRL-TK Renilla reporter constructs. In addition, 1.5 mg of CMV5-empty vector or 1.5 mg of CMV5-DLEU7-HA was used. In addition, 100 ng of CMV6-CD40, CMV6-TRA5, or a combination of both were used where indicated. Firefly and Renilla luciferase activities were assayed with the Dual-Luciferase Assay System from Promega, and firefly luciferase activity was normalized to Renilla luciferase activity. The normalized promoter activity of pNF-κB-Luc in HEK 293 cells transfected with CMV5-empty vector was set as 1. Experiments were repeated 3 times in triplicate. Columns indicate the mean; bars, SD.
activity induced by overexpression of TRAF6, CD40, and both TRAF6 and CD40. No such effect was observed when NF-κB was activated by TRAF5 (data not shown).

A previous study described a transgenic mouse line specifically expressing APRIL (a proliferation-inducing ligand) in lymphoid cells. An assessment of 9- to 12-month-old transgenics by flow cytometric analysis revealed significant expansions of B220+CD5+ cells in mesenteric lymph nodes and Peyer patches. The incidence and severity of these alterations increased over time, suggesting progressive expansion of mature CD5+ B cells, a disease similar to human CLL. APRIL, and its close relative BAFF are 2 recent members of the TNF superfamily, expressing almost exclusively in hematopoietic cells. Both BAFF and APRIL show elevated expression levels in patients with various B-cell malignancies, including diffuse large-cell lymphoma, mantle cell lymphoma, and CLL. APRIL binds with high affinity to 2 receptors, BCMA (B-cell maturation antigen) and TACI. BCMA is detected in mature B and T cells, whereas TACI is mostly expressed in activated T cells and subpopulations of B cells. BCMA and TACI are members of the TNF receptor superfamily. They interact with various TRAFs and stimulate the NF-κB pathway. All these data suggest that NF-κB activation through TACI and BCMA is important in the pathogenesis of CLL. Because we found that Dleu7 functions as an inhibitor of NF-κB activation by the TNF pathway, we investigated whether Dleu7 expression has an effect on NF-κB activation by TACI and BCMA.

To test this hypothesis, we carried out experiments similar to that described in Figure 2B. HEK 293 cells were transfected with constructs indicated on Figure 3A (left). BCMA expression resulted in 152-fold activation, which was inhibited by Dleu7 more than 5-fold (Figure 3A left). Similarly, Figure 3A (left) shows that Dleu7 expression inhibited NF-κB activation by TACI more than 4-fold. Thus, these data clearly indicate that Dleu7 functions as an NF-κB inhibitor in a pathway critical for the pathogenesis of CLL.

NFAT can be activated by TACI and BCMA. Previous reports showed that nuclear NFAT is a hallmark of unstimulated CLL cells, and that NFAT is necessary for CD5 expression in B cells. Since Dleu7 inhibits NF-κB activation by TACI and BCMA, we investigated whether Dleu7 can inhibit the ability of TACI and BCMA to induce NFAT transactivating function. To determine whether Dleu7 expression affects the transactivating activity of NFAT, we used a commercial NFAT reporter construct, pNFAT-Luc, expressing luciferase under the control of an NFAT-responsive element. HEK 293 cells were transfected with constructs indicated in Figure 3A (right). NFAT alone activated the reporter approximately 18-fold, whereas expression of BCMA increased this activation to approximately 400-fold. Dleu7 expression inhibited this activation approximately 8-fold. Similar inhibition (~8-fold) was observed when we used TACI to induce NFAT-dependent activation (Figure 3A right). Thus, we conclude that Dleu7 functions as NFAT inhibitor. Because TACI and BCMA activate NFAT by a different mechanism from that used for NF-κB activation, it is reasonable to speculate that Dleu7 functions as a TACI and BCMA inhibitor by direct association with these molecules.

To determine intracellular localization of Dleu7 and its complexes with TACI and BCMA, we carried out immunofluorescence experiments. NIH-3T3 cells were used since B-CLL cell lines practically do not exist, and NIH-3T3 cells are commonly used for intracellular localization studies because of their morphology. NIH-3T3 cells were transfected with the indicated constructs. Figure 4 shows that Dleu7 alone mostly localized in the cytoplasm with weaker nuclear staining, TACI and BCMA also show cytoplasmic localization and significant presence at the plasma membrane. Figure 4 (bottom) shows that Dleu7-TACI and Dleu7-BCMA complexes (yellow) localized mostly in the cytoplasm; some colocalization is also evident at the plasma membrane. These data serve as additional evidence that Dleu7 inhibits TACI and BCMA function by direct association.

Since we proved that Dleu7 inhibits NF-κB and NFAT-dependent transcription and inhibits TACI and BCMA function by direct association, it is reasonable to speculate that Dleu7’s actions will result in increased cell death. Because there are very few CLL cell lines, we used A549 lung cancer cells extensively used

**Figure 3. Interaction of Dleu7 with members of the TNFR superfamily, BCMA and TACI, interferes with NF-κB and NFAT transactivation. (A)** HEK293 cells were cotransfected with 50 ng of pNF-κB-Luc reporter or 250 ng of pNFAT-Luc reporter, respectively, and 50 ng of pRL-TK Renilla reporter constructs. In addition, 1.5 μg of CMV5-empty vector or 1.5 μg of CMV5-DLEU7-HA was used. In addition, 250 ng of CMV6-BCMA and 25 ng of CMV6-TACI (in the case of NF-κB-Luc) and 5 ng of CMV-NFAT, 100 ng of CMV6-BCMA and 5 ng of CMV6-TACI (in the case of NFAT-Luc) were used where indicated. Firefly and Renilla luciferase activities were assayed with the Dual-Luciferase Assay System from Promega, and firefly luciferase activity was normalized to Renilla luciferase activity. The normalized promoter activity of pNF-κB–Luc and pNFAT-Luc in HEK 293 cells transfected with CMV5-empty vector was set as 1, respectively. Experiments were repeated 3 times in triplicate. Columns indicate the mean; bars, SD. (B) Left panels show HEK 293 cells cotransfected with GLTX5-HA (negative control) and TACI-Myc, or hDLEU7-HA and TACI-Myc constructs. After lysis, immunoprecipitations were carried out using anti-HA antibody, mouse immunoglobulin G, or anti-Myc antibody. Western blotting was carried out as indicated. Right panels show HEK 293 cells cotransfected with BCMA-myc and FHIT-GFP (negative control), or BCMA-Myc and hDLEU7-GFP constructs. After lysis, immunoprecipitations were carried out using anti-GFP antibody, mouse immunoglobulin G, or anti-Myc antibody. Western blotting was carried out as indicated.
## Discussion

The 13q14 deletion is the most common CLL aberration and is observed by cytogenetics in approximately half of the cases and by loss of heterozygosity (LOH) in approximately 70% of cases. Previous reports demonstrated that the miR-15a/miR-16-1 cluster is located within the deleted region and showed a reduction in expression of miR-15a/miR-16-1 in most CLL cases. A subsequent report demonstrated that miR-15/16 targets Bcl2 expression, contributing to the pathogenesis of CLL. A recent report described a high-resolution map of 13q14 and showed that DLEU7 is the only protein-coding gene located within the minimal deleted region at 13q14. This prompted us to investigate whether DLEU7 can function as a tumor suppressor. Our results clearly show that Dleu7 functions as a potent inhibitor of NF-κB signaling by inhibiting TACI and BCMA, critical transducers of proliferative signals in B cells. All 3 known mouse models of CLL use abnormal activation of the NF-κB pathway. Eμ-TCL1 mice abnormally...
express Tcl1 in mouse B cells, and Tcl1 functions as an activator of NF-κB–dependent transcription.14,23 The APRIL transgenic mouse model induces NF-κB signaling through the TNF pathway using TACI and BCMA as transducers.13 Finally, the BCL2/TRA2FDN mouse model of B-CLL also uses NF-κB because TRAFs are important transducers of NF-κB signaling.24 The importance of our results is greatly enhanced by the fact that both APRIL and BCL2/TRA2FDN CLL mouse models use the same pathway: transduction NF-κB–activating signals through TACI and BCMA.

The BCL2/TRA2FDN transgenic mouse model represents a very interesting parallel and a confirmation of our findings. Bcl2 transgenic mice were produced by using a construct which mimics t(14;18) translocation, juxtaposing the BCL2 oncogene with the immunoglobulin heavy-chain locus at 14q32 observed in human follicular lymphomas. These mice did not develop any tumor phenotype until very late in life and did not show an increase in the B220+CD5− cell population, but showed polyclonal expansions of B cells and prolonged B-cell survival in vitro.25 TRAF2 transgenics and zinc finger domains located at the N-terminal of the molecule B signaling.24 The importance of our mouse model of B-CLL also uses NF-κB–activating signals through TACI and BCMA.

Deletions at 13q14 result in the loss of APRIL resulting in activation of NF-κB through TRAFs. Cooperation of these 2 events results in the pathogenesis of CLL.

Figure 6. Schematic representation of Dleu7 tumor-suppressor function in B cells.

DLEU7, 2 tumor suppressors. miR-15/16 deletions cause Bcl2 overexpression, and inactivation of DLEU7 causes induction of TNF signaling through TRAFs. Cooperation of these 2 events results in the pathogenesis of CLL.

Acknowledgments

The research was supported by an American Cancer Society Research Scholar grant (Y.P.) and National Institutes of Health grant PO1-CA81534 of the CLL Research Consortium (L.R., T.K., and C.M.C.).

Authorship

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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