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Epigenetic alterations in a murine model for chronic lymphocytic leukemia

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Early stages in the development of chronic lymphocytic leukemia (CLL) have not been explored mainly due to the inability to study normal B-cells en route to transformation. In order to determine such early events of leukemogenesis, we have used a well established mouse model for CLL. Overexpression of human *TCL1*, a known CLL oncogene in murine B-cells leads to the development of mature CD19$^+$/CD5$^+$/IgM$^+$ clonal leukemia with a disease phenotype similar to that seen in human CLL. Herein, we review our recent study using this TCL1-driven mouse model for CLL and corresponding human CLL samples in a cross-species epigenomics approach to address the timing and relevance of epigenetic events occurring during leukemogenesis. We demonstrated that the mouse model recapitulates the epigenetic events that have been reported for human CLL, affirming the power and validity of this mouse model to study early epigenetic events in cancer progression. Epigenetic alterations are detected as early as three months after birth, far before disease manifests at about 11 months of age. These mice undergo NFκB repressor complex mediated inactivation of the transcription factor Foxd3, whose targets become aberrantly methylated and silenced in mouse and human CLL. Overall, our data suggest the accumulated epigenetic alterations during CLL pathogenesis as a consequence of gene silencing through TCL1 and NFκB repressor complex, suggesting the relevance for NFκB as a therapeutic target in CLL.

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Introduction

CLL is characterized by several genetic abnormalities and certain laboratory tests can predict rapid disease progression and shortened survival.[1,2] Progression from early stage CLL to refractory disease is commonly associated with clonal evolution, as defined by multiple genetic abnormalities.[3,4] While long-term longitudinal follow-up of CLL patients is part of many studies, no study has effectively identified early initiating features in CLL. Identification of one or more initiating events that occur prior to development of multiple genetic abnormalities could provide insight into the etiology of CLL and also establish the rationale for pharmacologic targeting to prevent the development or progression of this disease.

Epigenetic Alterations in Human CLL

Epigenetic alterations in cancer cell genomes have been recognized as a major contributor to malignant phenotypes for several types of cancer. Epigenetic alterations do not change the DNA sequence and yet are heritably transmitted to daughter cells. Two main epigenetic alterations, DNA methylation and modifications of chromatin proteins, have been described. These epigenetic alterations are interrelated and it is thought that they

cooperate in the activation or silencing of genes through changes in chromatin conformation and accessibility (reviewed in ref. 5). The role of aberrant DNA methylation in CLL is not clear.[6] In a global screen for CpG island methylation, DNA hypermethylation was found in CLL patients with a mean of 4.8% of CpG islands affected.[7] These genes included novel tumor suppressor genes, such as *DAPK1*, *SFRP1*, *ID4* and genes involved in apoptosis.[8-11] Additionally, cell cycle regulators *CDKN2A*, *CDKN2B*,[12-14] and prognostic markers *ZAP70* and *TWIST2* have been found to be methylated in CLL patients.[15,16]

Early Epigenetic Events in the Progression of CLL in a Mouse Model

Two major questions in CLL pathogenesis emerging now are: what is the relevance of gene silencing events in the leukemic process? And, are these events required for transformation into a malignant clone, or are these events secondary ones that accumulate during leukemogenesis? To address these important questions, we determined the early events in malignant progression under the assumption that these events could represent required epigenetic changes. However, there is a lack of defined early pre-leukemic stage human CLLs. Given the similarity of the Eμ-TCL1 transgenic mouse model of CLL to the human disease of CLL,[17,18] we performed a comprehensive epigenetic study with TCL1 mice during the time period of polyclonal/oligoclonal pre-malignant cell expansion to seek early changes and potential targets for therapy. In these mice, initial expansion of non-clonal B lymphocytes occurs at approximately three months and is followed by progression to a mature B-cell leukemia at 9–11 months, with immuno-phenotypic and clinical characteristics of human CLL.

We performed a genome-wide scan for aberrant CpG island methylation in B-cells collected at multiple time points during the progression to CLL and detected aberrant DNA methylation in cells harvested three months after birth, a time when no disease phenotype was visible. DNA methylation levels increased from 0.4%, 0.6%, 1.2% and 1.9% (at 3, 5, 7 and 9 months, respectively) to 3.9% in Eμ-TCL1 mice with advanced CLL.[19] Most interestingly, the identified epigenetic target genes were comparable to those identified in human CLL. We tested ten of the early targets identified in the mouse model and found nine of them methylated and silenced in human CLL. The similarity of this murine CLL model and human CLL methylation patterns therefore provides further justification for using this system.

In addition to DNA hypermethylation, previous tumor studies reported that an overall decrease in 5-methylcytosine levels arising from hypomethylation of normally methylated repetitive elements which may also contribute to tumorigenesis. Therefore, we analyzed whether global hypomethylation occurs in the CLL cells of Eμ-TCL1 mice. The proviral sequences related to the intracisternal A particle (IAP) and centromeric repeat sequences were used as probes for methylation analysis of the repetitive sequences by Southern blot. After the comparison of *Hpa*II (methylation sensitive) and *Msp*I (methylation insensitive) DNA digests, we determined that IAP (**Fig. 1**, top) and centromeric repeat sequences (**Fig. 1**, middle) were heavily methylated in 4 and 11 months old wild type C3H/B6 mice; but hypomethylated in Eμ-TCL1 mice as early as 7 months old. Moreover, hypomethylation of LINE1 repeat elements was also increased in relapsed CLL patients with advanced disease as compared to primary untreated patients with early disease (**Fig. 1**, bottom).

Elevated DNA Methylation in CLL Follows the Pattern of de novo DNA Methyl-Transferase Activity

DNA methylation is mediated by transferring a methyl group from the methyl donor, S-adenosyl-methionine, to position 5 of the cytosine ring in DNA. This reaction is catalyzed by DNA methyltransferases (DNMT).[20] De novo methyltransferases (i.e., DNMT3A, 3B and 3L) establish the initial DNA methylation pattern within the genome,[21] whereas the maintenance enzyme (DNMT1) maintains the methylation pattern during DNA replication.[22] In Eμ-TCL1 mice we have observed the lack of initiating DNA methyltransferases, DNMT3A/3B, during the genesis of transformation but increasing protein levels of DNMT3A/3B at later stages. In contrast to protein expression, no significant changes were noted in the mRNA expression levels of these enzymes in $CD19^+$ splenocytes from Eμ-TCL1 at any stage. These data suggest a posttranscriptional regulation of DNMT3A/3B might be achieved by microRNAs, similar to recent findings in lung cancer, where miR29s has been identified to regulate de novo DNA methyltransferases DNMT3A/3B.[23] Indeed, decreased expression of miR29B and miR29C (but not miR29A) was noted in TCL1 mice at 5 and 7 months of age (**Fig. 2**), corresponding with the increased DNMT3A/3B protein expression at later ages and is consistent with the notion of direct DNMT3A/3B regulation by the low-expressed miR29s in CLL B-cells.[24] The increased activity of DNMT3A/3B may be one of the factors needed to increase CpG island hypermethylation, as described above.

Silencing of Foxd3 is a Key Event in Progression to CLL

The fact that accumulating epigenetic alterations in specific CpG island genes starts from a small number of changes allowed us to consider identifying key initiating factor(s). For this purpose, we sought to identify a conserved domain specifically located within early methylated promoters.[19] The results show that 70% of the methylated genes in Eμ-TCL1 mice are putative targets of the transcriptional activator *FOXD3*, a gene that was found methylated in TCL1 mouse B-cells prior to malignant transformation. FOXD3 is a member of the fork head-box (FOX) family of transcription factors that is characterized by a 100 amino acid monomeric DNA binding domain, which is highly conserved among all FOX proteins for nuclear localization and transcriptional regulation.[25-27] FOXD3 plays a crucial role in gene regulation and is involved in a tight regulatory feedback loop with OCT4 and NANOG. The interactions and balanced expression of factors in this negative feedback loop formed by FOXD3, OCT4 and NANOG are essential in maintaining

the multipotent properties of embryonic stem cells.[28-31] In B lymphocytes, FOXD3 promoter activity was determined to be negatively regulated by TCL1 in the WAC3CD5 CLL cell line (**Fig. 3A**). Silenced *FOXD3* was also noted in CLL samples with high TCL1 expression (**Fig. 3B**). The mechanism of gene deregulation involving the transcription factor FOXD3 in B cells was then suggested by demonstrating TCL1-mediated transcriptional silencing of FOXD3 through a novel NFκB p50/p50, histone deacetylase 1 (HDAC1) co-repressor complex. All together, our evidence suggests that silencing of transcription factors, such as FOXD3, at an early time point in the preclinical phase of disease initiation may be responsible for early transcriptional and later epigenetic silencing of multiple genes involved in the transformation of mouse, and possibly human, CLL.[19]

Pre-Leukemic Stages in Human CLL

Recent studies identified small clones of B-cells using six-color flow cytometry with antibodies against CD45, CD19, CD5, CD10, kappa and lambda light chains, in addition to detecting immunoglobulin heavy chain gene rearrangements, in healthy individuals with no signs of a lymphoproliferative disorder. The number of monoclonal B-cells in these clusters were below 5,000 per cubic millimeter.[32] This newly described condition was termed monoclonal B-cell lymphocytosis, or MBL, and has now been shown to be a precursor for CLL. The frequency of MBL ranges from 3–5% in the general population. Interestingly, the majority of CLL patients (44 out of 45 individuals studied by Landgren et al.) showed this phenotype.[33] Study samples were collected up to 77 months before the diagnosis of CLL, thus arguing that MBL is a precursor stage of CLL. In the future it might be possible to utilize these samples for the discovery of early events in the progression to human CLL. However due to the small number of individuals presenting with MBL (1% of the population), and the infrequent progression to CLL, the mouse model presents a more robust study system at this time.

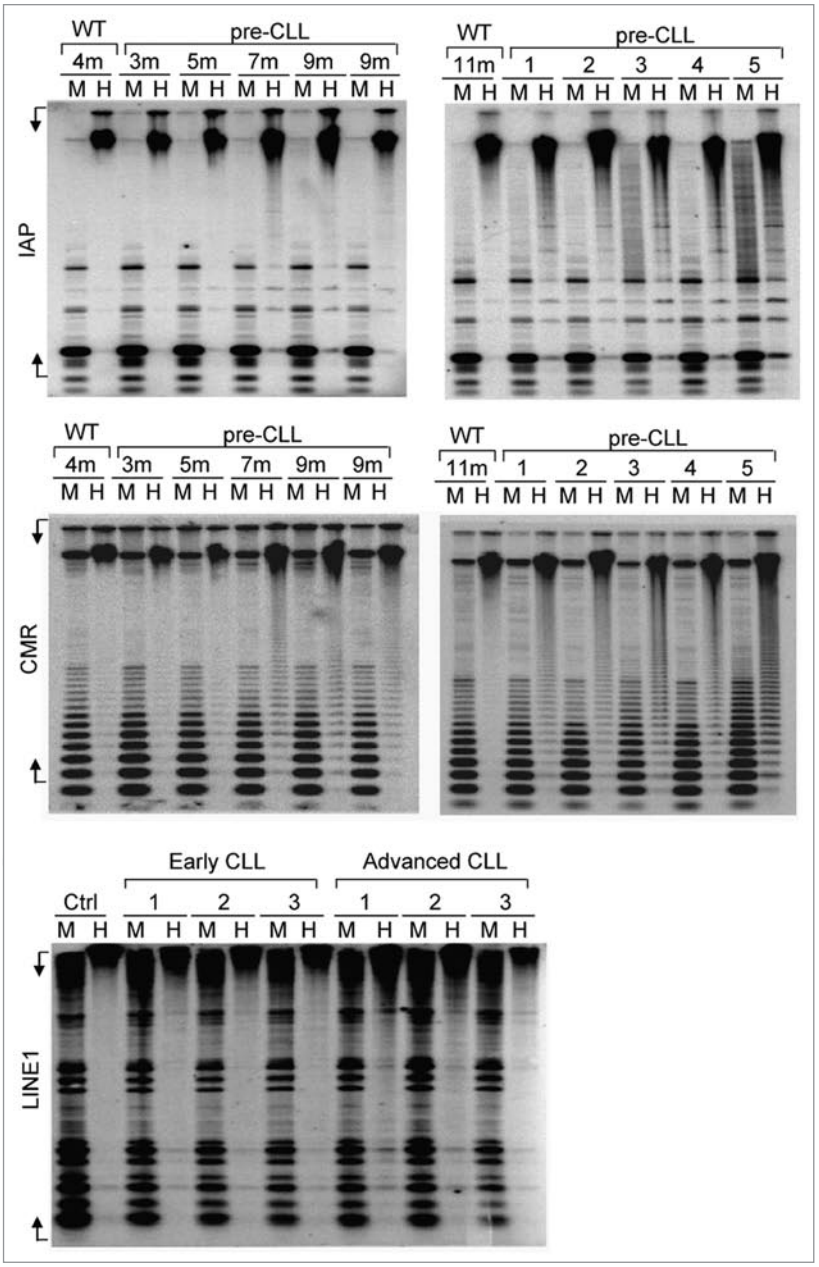

Figure 1. Elevated global DNA hypomethylation of repetitive sequences in CLL. DNA hypomethylation profiles for mouse and human CLL samples from different stages were studied by Southern blotting as previous described.[34] Genomic DNAs of spleen cells from wild type and Eμ-TCL1 mice at the indicated time points, or Eμ-TCL1 mice with symptomatic disease (n = 5), were digested with *Msp*I (M) or *Hpa*II (H) restriction enzymes. The blots were hybridized with intracisternal A particle probe (IAP) (Top) or centromeric repeat sequences (CMR) (Middle). For CLL patient samples, the analysis was done using human LINE1 probes on peripheral blood B cells from patients who required no treatment (early or indolent CLL) or that underwent treatment (advanced CLL) (Bottom). *Msp*I digested DNA was used as a control. The undigested product of *Hpa*II digestion represents methylated DNA, whereas only unmethylated DNA is *Hpa*II digestible. Mouse IAP, CMS and human LINE1 probes were prepared by PCR amplification using forward (F) and reverse (R) primers: IAP-F: 5'CGT CAT TGT TCA GAG CCA GA3', IAP-R: 5'TCC CGG AAA CTT TTG TTC AC3'; CMS-F: 5'GAT AAA AAC CTA CAC TGT AG3', CMS-R: 5'GTT TCT AAT TGT AAC TCA TTG3'; LINE1-F: CGG GTG ATT TCT GCA TTT CC and LINE1-R: GAC ATT TAA GTC TGC AGA GG.

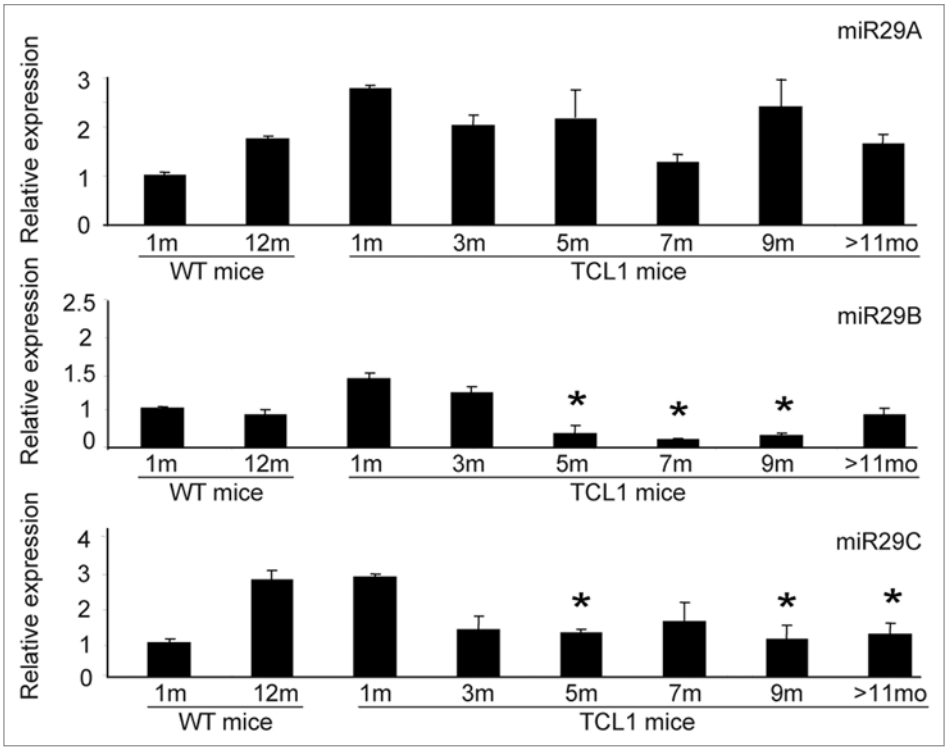

Figure 2. Decreased miR29 and increased DNMT expression in Eμ-TCL1 mice. Expression of miR29s by TaqMan PCR was done for TCL1 mouse spleen B cells. Each bar represents the average result from three mice at the indicated age. Results were normalized by the data obtained from untransformed B cells from 1 month old mice. Standard deviations were calculated using the mean ± SEM for the respective data. A two-tail unequal variant student T test was applied to the data; a star represents significant results with $p < 0.05$.

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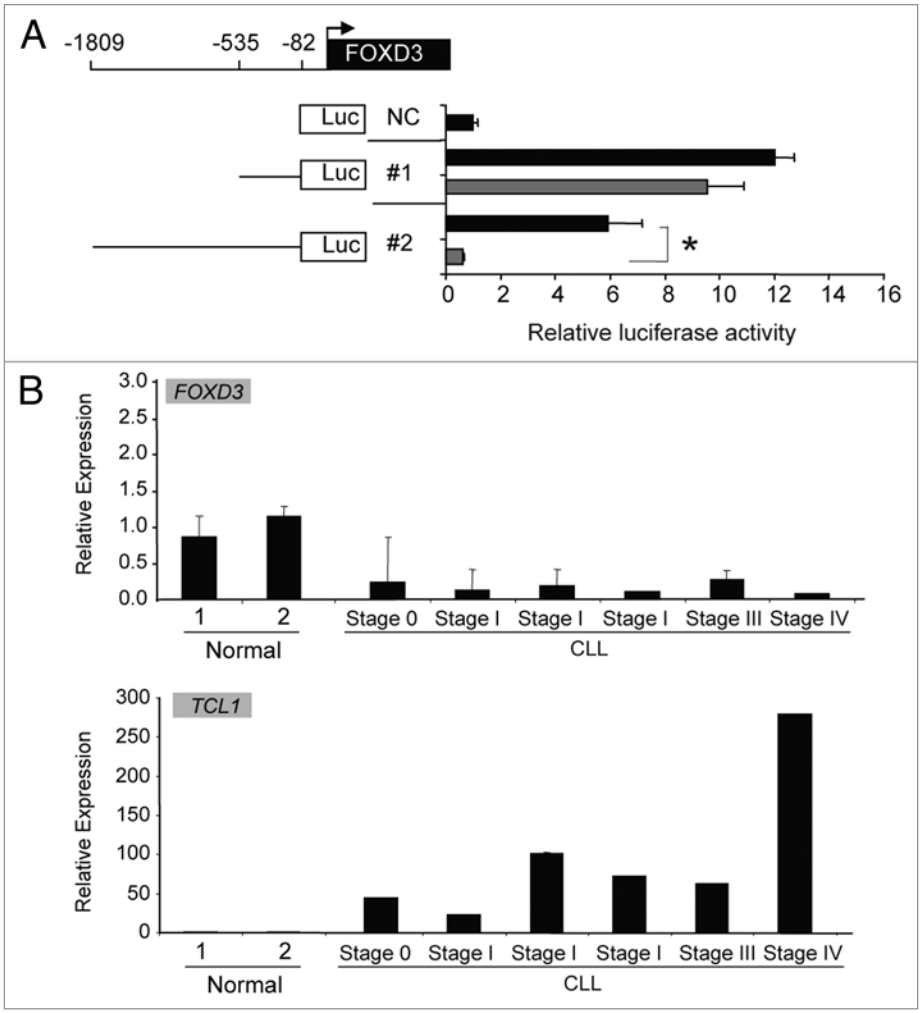

Figure 3. FOXD3 repression by TCL1 in CLL cells. (A) A FOXD3-promoter luciferase-reporter assay was done in the WAC3CD5 cell line transfected with 1 μg pCMV-TCL1 (gray bar) or a control vector (black bar). The diagram shows the human *FOXD3* 5' promoter region cloned into a pGL3 vector for analysis. Results from each cell line co-transfected with pGL3-basic vector (NC) were normalized and set to 1.0. Each bar represents the average result from triplicate experiments using the mean ± SEM of the respective data. (B) *FOXD3* and *TCL1* expression were analyzed in two normal B cell samples and 6 CLL B cell samples from patients with different diagnoses. The error bars represent the mean ± SEM of the respective data from triplicate experiments.

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