Association of a MicroRNA/TP53 Feedback Circuitry With Pathogenesis and Outcome of B-Cell Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia among adults in the Western world, with an annual incidence in the United States of approximately 10,000 new cases. The clinical staging systems devised by Rai et al and Binet et al are useful for assessing the extent of CLL in a patient, but they fail to differentiate between the indolent and aggressive forms of CLL. Most typically these forms are characterized by low and high levels of zeta-chain (TCR)–associated protein kinase 70kDa (ZAP70), a surrogate prognostic marker of CLL. The functional relationship between these genes was studied using in vitro gain- and loss-of-function experiments in cell lines and primary samples and was validated in a separate cohort of primary CLL samples.

Context Chromosomal abnormalities (namely 13q, 17p, and 11q deletions) have prognostic implications and are recurrent in chronic lymphocytic leukemia (CLL), suggesting that they are involved in a common pathogenetic pathway; however, the molecular mechanism through which chromosomal abnormalities affect the pathogenesis and outcome of CLL is unknown.

Objective To determine whether the microRNA miR-15a/miR-16-1 cluster (located at 13q), tumor protein p53 (TP53, located at 17p), and miR-34b/miR-34c cluster (located at 11q) are linked in a molecular pathway that explains the pathogenetic and prognostic implications (indolent vs aggressive form) of recurrent 13q, 17p, and 11q deletions in CLL.

Design, Setting, and Patients CLL Research Consortium institutions provided blood samples from untreated patients (n=206) diagnosed with B-cell CLL between January 2000 and April 2008. All samples were evaluated for the occurrence of cytogenetic abnormalities as well as the expression levels of the miR-15a/miR-16-1 cluster, miR-34b/miR-34c cluster, TP53, and zeta-chain (TCR)–associated protein kinase 70kDa (ZAP70), a surrogate prognostic marker of CLL. The functional relationship between these genes was studied using in vitro gain- and loss-of-function experiments in cell lines and primary samples and was validated in a separate cohort of primary CLL samples.

Main Outcome Measures Cytogenetic abnormalities; expression levels of the miR-15a/miR-16-1 cluster, miR-34 family, TP53 gene, downstream effectors cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A) and B-cell CLL/lymphoma 2 binding component 3 (BBC3), and ZAP70 gene; genetic interactions detected by chromatin immunoprecipitation.

Results In CLLs with 13q deletions the miR-15a/miR-16-1 cluster directly targeted TP53 (mean luciferase activity for miR-15a vs scrambled control, 0.68 relative light units (RLU) [95% confidence interval (CI), 0.63-0.73]; P=.02; mean for miR-16 vs scrambled control, 0.62 RLU [95% CI, 0.59-0.65]; P=.02) and its downstream effectors. In leukemic cell lines and primary CLL cells, TP53 stimulated the transcription of miR-15/miR-16-1 as well as miR-34b/miR-34c clusters, and the miR-34b/miR-34c cluster directly targeted the ZAP70 kinase (mean luciferase activity for miR-34a vs scrambled control, 0.33 RLU [95% CI, 0.30-0.36]; P=.02; mean for miR-34b vs scrambled control, 0.31 RLU [95% CI, 0.30-0.32]; P=.01; and mean for miR-34c vs scrambled control, 0.35 RLU [95% CI, 0.33-0.37]; P=.02).

Conclusions A microRNA/TP53 feedback circuitry is associated with CLL pathogenesis and outcome. This mechanism provides a novel pathogenetic model for the association of 13q deletions with the indolent form of CLL that involves microRNAs, TP53, and ZAP70.
Patients, Age, mean (SD), y

Table. Clinical Data From All Patients With B-Cell Chronic Lymphocytic Leukemia in Molecular Studies

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>13q Alone</th>
<th>11q Alone</th>
<th>17p Alone</th>
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<tbody>
<tr>
<td>Male/Female</td>
<td>188</td>
<td>12/6</td>
<td>10/5</td>
<td>0/0</td>
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<tr>
<td>Age (years)</td>
<td></td>
<td>61.2</td>
<td>57.4</td>
<td>55.8</td>
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<tr>
<td>Deletion (n = 18)</td>
<td></td>
<td>28</td>
<td></td>
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</tr>
</tbody>
</table>

Aberration ZAP70 expression (TP53-associated protein kinase 70kDa)

Additional Partial Chromosomal Abnormalities (% of Cases)

<table>
<thead>
<tr>
<th>Deletion (n = 18)</th>
<th>11q Alone</th>
<th>17p Alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>17p</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>11q</td>
<td>12</td>
<td>10</td>
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<tr>
<td>13q</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>17p and 11q</td>
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<td>4</td>
</tr>
<tr>
<td>11q and 17p</td>
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<tr>
<td>13q and 17p</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>13q, 11q, and 17p</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

Table continues...
(wild-type TP53) are commercially available for the Tet-Off Advanced Inducible Gene Expression System (Clontech, Mountain View, California) (see eMethods for details). The efficiency of transfection for cell lines and primary B-CLL cells was checked by quantitative real-time polymerase chain reaction (qRT-PCR) analysis (eFigure 1).

**Genes**

Two genes were considered downstream effectors of the TP53 pathway signaling: cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A [GenBank 1026]), the expression of which is tightly regulated by TP53, which induces a TP53-dependent cell cycle G1 phase arrest in response to various stress stimuli; and BCL2 binding component 3 (BBC3 [GenBank 27113], formerly named PUMA, a p53 upregulated modulator of apoptosis, which binds to BCL2 and is a proapoptotic gene). Procaspase 3 is the precursor of caspase 3 apoptosis-related cysteine peptidase (CASP3), a key protein involved in apoptosis. The cleavage of procaspase 3 to CASP3 is one of the strongest indicators of apoptosis.

**Luciferase Reporter Assays**

MicroRNAs interact with their target genes by means of a “seed” region sequence between the mature microRNA and the target gene messenger RNA (mRNA). A luciferase reporter assay is performed to demonstrate that the microRNA-mRNA interaction is direct (meaning by complementarity). In this assay, the microRNA binding site on the target mRNA is cloned on a plasmid carrying the gene for luciferase, just downstream of the luciferase gene. Cells are then cotransfected with this plasmid and the microRNA of interest (or a scrambled microRNA as a control). If the microRNA targets the cloned binding site, the overall luciferase reporter activity in the cells cotransfected with the microRNA of interest is reduced. The destruction (and/or mutation) of the microRNA binding

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**Figure 1.** Targeting of TP53 by miR-15a and miR-16 and Effects on TP53 Downstream Effectors in Cell Lines and Primary B-Cell Chronic Lymphocytic Leukemia (B-CLL) Samples

A, Immunoblots showing the protein expression of tumor protein p53 (TP53), B-cell CLL/lymphoma 2 (BCL2), and vinculin (VCL) in MEG-01 cells transfected with microRNA 15a (miR-15a), microRNA 16 (miR-16), their combination, or their antisense oligonucleotides. Cotransfection of miR-15a and miR-16-1 was performed at the same concentration of oligonucleotides per each; therefore, the total amount of transfected microRNAs was doubled with respect to the other lanes. VCL is the normalization standard used to normalize the amount of proteins loaded to each well. The numbers above the blots indicate the intensity of the band expressed as a ratio “gene product (TP53 or BCL2)/VCL” and normalized to “scrambled.” B, Immunoblots showing the protein expression of TP53, cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), BCL2 binding component 3 (BBC3), BCL2, zeta-chain (TCR)-associated protein minuse 70 kDa (ZAP70), and VCL in primary B-cell CLL cells of 3 patients with CLL with a homozygous 13q deletion. Primary leukemic cells were stably infected with a lentiviral vector expressing miR-15a (LV-miR-15a), a lentiviral vector expressing miR-16-1 (LV-miR-16), or an empty lentiviral vector (LV-Empty). VCL is the normalization standard used to normalize the amount of proteins loaded to each well. The numbers above the blots indicate the intensity of the band expressed as a ratio “gene product (TP53, CDKN1A, BBC3, BCL2 or ZAP70)/VCL” and normalized to “LV-Empty.”
site on the target mRNA abolishes the reduction in the luciferase reporter activity, which indicates that the targeting is direct. A luciferase reporter assay also was used to determine the effects of TP53 on the expression of the microRNAs of interest. TP53, like many other transcription factors, recognizes a specific binding sequence and binds to it. As a result, any gene located downstream of the binding site can be activated or suppressed. The predicted TP53 binding sites were cloned upstream of the luciferase gene in a luciferase-expressing reporter plasmid, and H1299 cells were transfected with this plasmid and a TP53-expressing plasmid or an empty plasmid. The effect (either activation or suppression) of TP53 on the expression of the microRNAs of interest was expressed as increased or decreased luciferase reporter activity of the TP53-treated group vs that of the empty vector–treated group (see eMethods for details).

**Assessment of ZAP70 and Cytogenetic Data**

Expression of ZAP70 was assessed by immunoblotting and flow cytometry analysis. Cytogenetic data were available for all 206 patients in this study. The following probes were used to perform FISH analyses: ataxia telangiectasia mutated (ATM) (11q22.3), D13S319 (13q14.3), and TP53 (17p13.1, henceforth designated 17p13). A commercial probe set (CLL Panel; Vysis Inc, Downers Grove, Illinois) was used to perform FISH analyses on peripheral blood samples that had been cultured for 24 hours without stimulation.

**Statistical Analysis**

Results are presented as means with 95% confidence intervals (CIs). A probability value of \(P < .05\) by 2-sided \(t\) test was considered statistically significant. Allelic distributions for the 3 chromosomal abnormalities (ie, 13q, 17p, and 11q deletions) in all patients were tested with a \(x^2\) goodness-of-fit test for compliance with Hardy-Weinberg equilibrium. Relationships between microRNA and TP53 or ZAP70 mRNA expression determined by qRT-PCR were calculated as Pearson correlations. Based on our previous qRT-PCR data for the expression of miR-15a and miR-16, we calculated that by using a co-
A, Northern blots showing messenger RNA (mRNA) expression of microRNA 16 (miR-16) and immunoblots showing the protein expression of tumor protein p53 (TP53), B-cell CLL/lymphoma 2 (BCL2), caspase 3, apoptosis-related cysteine peptidase (CASP3), and vinculin (VCL) in K562 leukemic cells 24 or 48 hours after transfection with an empty or TP53-expressing vector (TP53). VCL is the normalization standard used to normalize the amount of proteins loaded to each well. The numbers above the blots indicate the intensity of the band expressed as ratio “gene product (TP53, BCL2 or CASP3)/VCL” and normalized to “empty.” B, Immunoblots showing the protein expression of TP53 and VCL, and the Northern blots showing mRNA expression of miR-16 and RNA, U6 small nuclear 1 (RNU6-1), after doxorubicin-induced TP53 activation in MEG-01 cells. VCL and RNU6-1 are the normalization standards used to normalize the amount of proteins and RNA loaded to each well, respectively. The numbers above the blots indicate the intensity of the band expressed as ratio “TP53/VCL” or “miR-16/RNU6-1” and normalized to untreated cells (left panels) and to anti-CTRL treated cells (right panels).

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Luciferase reporter assay (as means [error bars indicate 95% confidence intervals] of experiments conducted in sextuplicate) in MEG-01 cells cotransfected with wild-type zeta-chain (TCR)-associated protein kinase 70kDa (ZAP70) binding site for microRNA 34 (miR-34) family wt and miR-34a, miR-34b, or miR-34c. Luciferase activity normalized to scrambled; RLU indicates relative light units. ZAP70 del indicates deletion of miR-34 binding site on ZAP70 coding region; ZAP70 mut indicates mutation of miR-34 binding site on ZAP70 coding region. P values calculated for miR-34a, miR-34b, and miR-34c vs scrambled; values were significant (P < .05) for ZAP70 wt (wild-type) only.

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directly to a pre-miR-34b/miR-34c TP53 binding site on chromosome 11 (eFigure 10 and eFigure 12). A luciferase reporter assay showed a statistically significant transactivating effect of TP53 on the miR-34b/miR-34c binding site (Figure 3A), which was confirmed in TP53-transfected MEG-01 cells, which had increased miR-34b expression levels (Figure 3B). Taken together, these results indicate that TP53 is a positive transcriptional regulator of the miR-34b/miR-34c cluster in leukemic cells, which is consistent with findings observed in epithelial cells.14

Patients with 11q+/− CLls (n=23) had significantly lower levels of miR-34b (mean fold induction, 0.37 [95% CI, 0.35-0.39]; P < .01) and miR-34c (mean fold induction, 0.39 [95% CI, 0.37-0.41]; P < .01) than patients with CLls with normal cytogenetic profiles (n=28) (set as fold induction, 1 [95% CI, 0.96-1.04] and 1 [95% CI, 0.97-1.03], respectively). Conversely, patients with 11q+/− CLls had significantly higher levels of ZAP70 than patients with CLls with normal cytogenetic profiles, both at the mRNA level (mean fold induction, 2.47 [95% CI, 2.43-2.51] vs 1 [95% CI, 0.94-1.06]; P=.005). Patients with 11q+/− CLls and high levels of ZAP70 experienced poorer overall survival than patients with CLls with normal cytogenetic profiles and lower levels of ZAP70 (mean, 72.4 [95% CI, 35.4-79.2] months vs 114.7 [95% CI, 98.9-130.5] months; P=.02). A binding site for the miR-34 family was detected in the ZAP70 opening reading frame (eFigure 13A). Reduced ZAP70 expression (both at the protein and mRNA level) was observed in primary B-CLL cells from a patient with an 11q+/− deletion, in which miR-34a, miR-34b, and miR-34c were overexpressed (eFigure 13B and eFigure 14).

No effect on cell growth and cell proliferation was observed up to 72 hours in MEG-01 (ZAP70-negative) cells or in K562 (ZAP70-positive) cells overexpressing miR-34b or miR-34c (eFigure 15). A luciferase reporter assay showed that all

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3 microRNAs directly target the predicted region on ZAP70 (mean luciferase activity, 0.33 RLU [95% CI, 0.30-0.36]; P=.02 for miR-34a, 0.31 RLU [95% CI, 0.30-0.32]; P=.01 for miR-34b, and 0.35 RLU [95% CI, 0.33-0.37]; P=.02 for miR-34c) and TP53, with 13q−/− CLL was associated with reduced expression levels of TP50, a tyrosine kinase relevant in the initial step of T-cell receptor-mediated signal transduction. Low expression levels of ZAP70 have been found to be positively correlated with survival in patients with CLL. Further explaining the indolent course of CLL carrying 13q deletions.

Here we showed that, in primary B cells from patients with B-CLL, use of viral infection to restore expression of the miR-15a/miR-16-1 cluster is associated with reduced expression levels of TP50, miR-34a, miR-34b, and miR-34c and increased protein levels of TP50. These findings also demonstrate that in primary B-CLLS, restoring the expression of a microRNA cluster (namely, the miR-15a/miR-16-1 cluster) indirectly affects the expression of another family of microRNAs (miR-34 family) by modulating the levels of TP50. Some of our results indicate that these effects also occur in non-CLL leukemic cells (such as the acute myelogenous leukemic cell line K562) and in nonhematologic cell lines (such as H1229, A549, and HeLa cells), which suggests that the proposed miRNA-TP53 loop is relevant to tumor types other than CLL. More studies are necessary to validate this statement.

In conclusion, we found that a microRNA/TP53 feedback circuitry is associated with the pathogenesis and prognosis of CLL. Our findings reveal a new pathogenetic model for human CLL that involves microRNAs (miR-15a/miR-16-1 and miR-34b/miR-34c) and protein-coding genes (such as TP53 and ZAP70) with well-known prognostic significance in CLL.

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REFERENCES


If humanity is to have a hopeful future, there is no escape from the preeminent involvement and responsibility of the single human soul, in all its loneliness and frailty.

—George F. Kennan (1904-2005)