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

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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*.

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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.[1] The clinical staging systems devised by Rai et al[2] and Binet et al[3] 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

See also p 95 and Patient Page.

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

	Deletion (n = 188)									Additional Deletion (n = 18)	
Characteristic	13q Alone	11q Alone	17p Alone	11q + 13q	17p + 13q	11q + 17p	11q + 17p + 13q	Normal Cytogenetic Profiles	Total	11q Alone	Total
Patients, No. (%)	115 (61.2)	5 (2.6)	5 (2.6)	18 (9.6)	15 (8.0)	0	2 (1.1)	28 (14.9)			
Men/women	68/47	4/1	3/2	12/6	10/5	0/0	1/1	19/9	117/71	14/4	131/75
Age, mean (SD), y	57.4 (14.6)	58.9 (12.3)	47.1 (10.4)	55.8 (13.7)	54.1 (15.1)	0	55.4 (4.3)	53.2 (9.5)	54.6 (12.1)	54.5 (10.4)	54.6 (11.3)
Rai stage											
0	60	2	4	5	6	0	1	6	84	7	91
1	39	2	1	10	1	0	0	18	71	9	80
2	10	1	0	3	4	0	1	3	22	2	24
3	2	0	0	0	3	0	0	1	6	0	6
4	4	0	0	0	1	0	0	0	5	0	5
Patients with ZAP70 positive cells >20%, No. (%)	28 (24.4)	2 (40.0)	2 (40.0)	12 (66.7)	8 (53.3)	0	2 (100)	12 (43.1)		10 (55.6)	

Abbreviation: ZAP70, zeta-chain (TCR)–associated protein kinase 70kDa.
[a]Measured by flow cytometry.

protein kinase 70kDa (ZAP70),[4,5] respectively. Using fluorescence in situ hybridization (FISH), Döhner et al[4] found that chromosomal abnormalities occurred in 82% of cases and included the 13q deletion (55%), 11q deletion (18%), and 17p deletion (7%). Patients with the 17p and 11q deletions experience the aggressive form of the disease, whereas patients with the 13q deletion or with normal cytogenetic profiles experience the indolent form.[4,6] The occurrence of common and recurring chromosomal abnormalities[4] suggests that these deletions affect thus-far undefined pathways important for the pathogenesis of CLL.

MicroRNAs are small, noncoding RNAs with regulatory functions[7]; microRNA expression is frequently deregulated in tumors.[8] In most CLLs, the expression of the microRNA 15a (*miR-15a* [GenBank 406948])/microRNA 16-1 (*miR-16-1* [GenBank 406950]) cluster, which maps within a 30-kilobase region of loss[9] at 13q14.3 (henceforth designated 13q14), is abolished or reduced.[10,11] We have previously shown that the expression of this microRNA cluster is inversely correlated with the expression of B-cell CLL/lymphoma 2 (*BCL2* [GenBank 596]), an antiapoptotic gene overexpressed in most CLLs.[12] The loss of the long arm of chromosome 11 involves the 11q23.1 region (henceforth designated 11q23), where the microRNA 34b (*miR-34b* [GenBank 407041])/microRNA 34c (*miR-34c* [GenBank 407042]) cluster is located.[13] The observation that microRNA 34a (*miR-34a* [GenBank 407040]), *miR-34b*, and *miR-34c* are transactivated by tumor protein p53 (TP53)[14] and that the *miR-34b/miR-34c* cluster maps at 17p suggests the possible existence of a genetic link and significant molecular interactions between the 17p and 11q chromosomal deletions in CLL. At present, it is not known how the 13q, 11q, and 17p deletions contribute to CLL pathogenesis and affect the outcome of patients with CLL.

METHODS

Patient Samples and Cell Lines

Blood samples were obtained at the CLL Research Consortium Institutions from 206 patients with B-cell CLL (TABLE). Written informed consent was obtained from all patients before sample analyses, and the study was in accordance with the approved institutional review board protocols (LAB07-0734 and 2005C0014).

We first evaluated the occurrence of chromosomal abnormalities in 188 consecutive patients with untreated CLL. Then, to test our hypothesis that the *miR-34b/miR-34c* cluster modulates *ZAP70* (GenBank 7535) expression in CLL, we analyzed 18 additional patients with B-CLL who had the 11q deletion alone. Details about the isolation of mononuclear cells are provided in the eMethods available at http://www.jama.com. MEG-01, K562, H1299, and A549 cell lines were obtained from the American Type Culture Collection.

Because of the lack of validated CLL cell lines, the megakaryocytic leukemia cell line MEG-01 was chosen because MEG-01 cells (wild-type *TP53* [GenBank 7157]) carry the 13q deletion, which is the chromosomal abnormality most frequently observed in CLL; thus, this cell line resembles the indolent form of CLL at the molecular level. K562 is a chronic myeloid leukemia cell line, chosen because K562 cells carry a mutated, inactive *TP53* and represent a model of chronic leukemia. H1299 is a lung cancer cell line with a homozygous deletion of the *TP53* gene, chosen to avoid interfering with endogenous TP53 in our experiments. A549 is a lung cancer cell line (wild-type *TP53*) chosen as a TP53-expressing control cell line for the H1299 model. HeLa is a cervical cancer cell line chosen because HeLa cells

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(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).

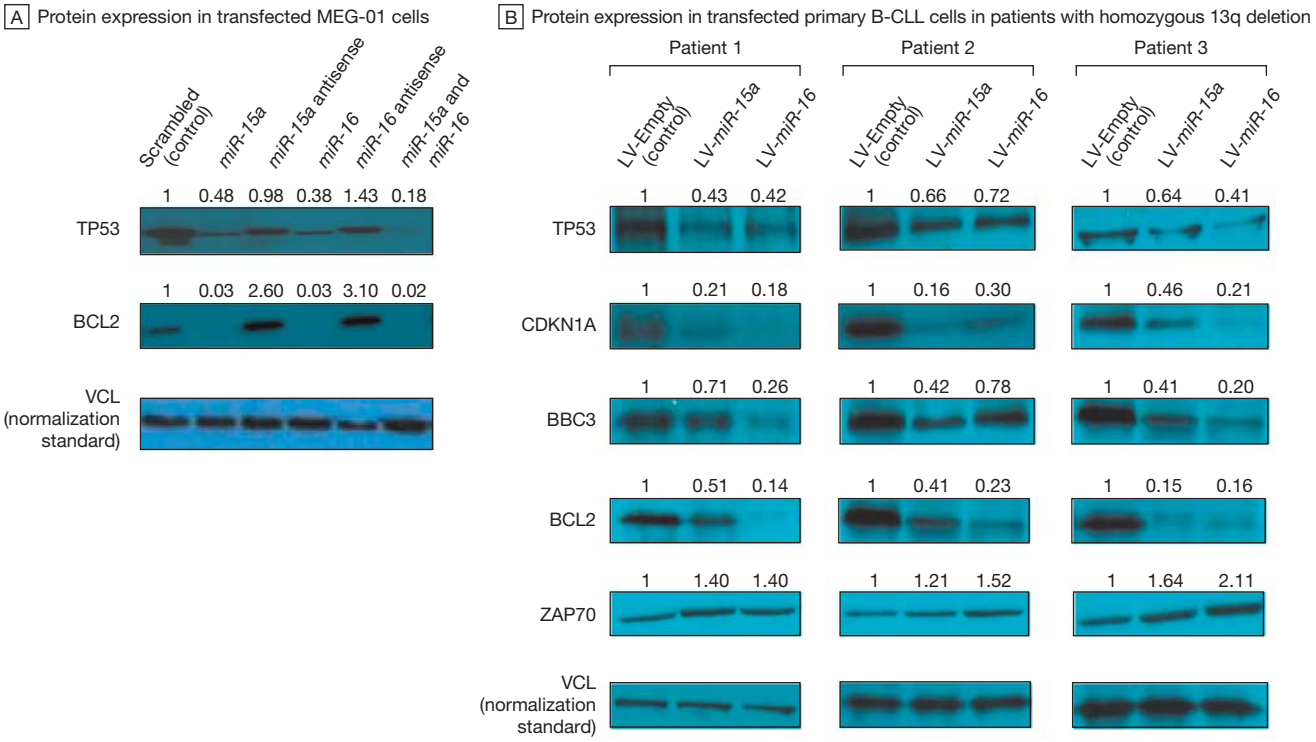
Genes

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

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, Cip 1) (*CDKN1A*), BCL2 binding component 3 (*BBC3*), *BCL2*, zeta-chain (TCR)–associated protein minase 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 ratio "gene product (TP53, CDKN1A, BBC3, BCL2 or ZAP70)/VCL" and normalized to "LV-Empty."

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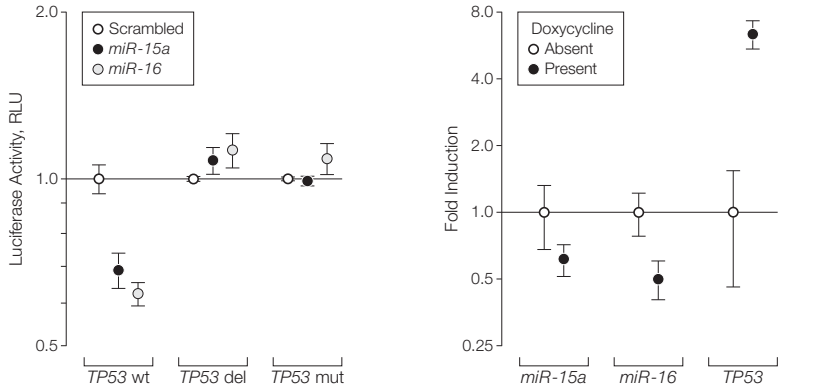
site on the target mRNA abolishes the reduction in the luciferase reporter activity, which indicates that the targeting is direct.

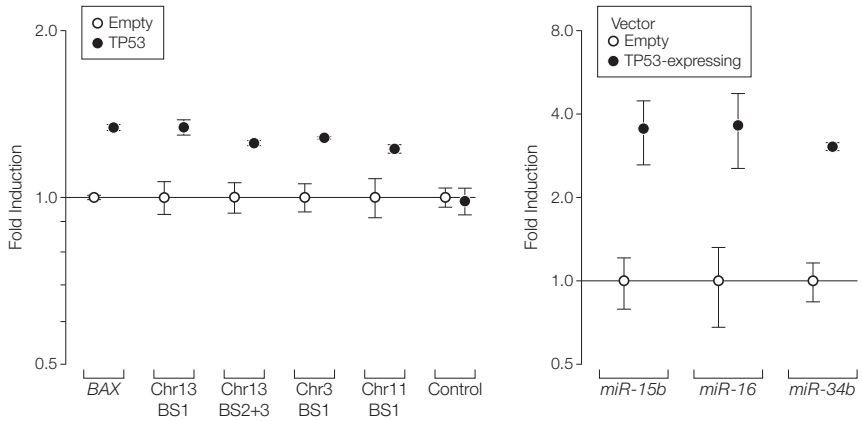
A luciferase reporter assay also was used to determine the effects of TP53 on the expression of the microRNAs of interest. TP53, like many other transcription factors, recognizes a specific binding sequence and binds to it. As a result, any gene located downstream of the binding site can be activated 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).

Figure 2. Targeting of *TP53* by *miR-15a* and *miR-16*



A, Luciferase reporter assay (as means [error bars indicate 95% confidence intervals] of experiments conducted in sextuplicate) in cells cotransfected with wild-type tumor protein p53 (*TP53*) 3′-UTR (*TP53* wt) and microRNA 15a (*miR-15a*) or 16 (*miR-16*). Luciferase activity normalized to scrambled; RLU indicates relative light units. *TP53* del indicates deletion of *miR-15a/miR-16* binding site on *TP53* 3′-UTR; *TP53* mut indicates mutation of *miR-15a/miR-16* binding site on *TP53* 3′-UTR. *P* values calculated for *miR-15a* and *miR-16* vs scrambled; values were statistically significant ($P<.05$) for *TP53* wt comparisons only. B, Expression of *miR-15a*, *miR-16*, and *TP53* messenger RNA (mRNA) in Tet-Off *miR-15a/miR-16-1*–inducible HeLa cells as detected by quantified real-time polymerase chain reaction. Results presented as means (error bars indicate 95% confidence intervals) of experiments performed in triplicate. *P* values calculated for the cells in the presence of doxycycline (indicates reduced expression of the *miR-15a/miR-16-1* cluster) vs the cells in the absence of doxycycline (indicates increased expression of the *miR-15a/miR-16-1* cluster); all values were statistically significant ($P<.05$).

Figure 3. Transactivation of *miR-15a/miR-16-1* and *miR-34b/miR-34c* Clusters by TP53



A, Promoter luciferase assay in tumor protein p53 (*TP53*)-null H1299 cells reported as means (error bars indicate 95% confidence intervals) of experiments performed in sextuplicate. The indicated TP53 binding site (BS) numbers correspond to those shown in red in eFigures 9 and 12. Control indicates the results obtained using the promoter vector with no binding site cloned in it. *BAX* indicates BCL2-associated X protein; Chr, chromosome. *P* values were calculated for TP53 vs empty for each group; all values were statistically significant ($P<.05$). B, Quantified real-time polymerase chain reaction (error bars indicate 95% confidence intervals) for microRNA 15b (*miR-15b*), microRNA 16 (*miR-16*), and microRNA 34b (*miR-34b*) performed on MEG-01 cells 24 hours after transfection with empty or TP53-expressing vectors. *P* values were calculated for TP53 vs empty for each group; all values were statistically significant ($P<.05$).

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 χ^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*,[15] we calculated that by using a co-

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hort of 206 patients, the statistical power would be greater than 95% to detect an effect, if one actually exists, with a minimal detectable effect of 9.73 (calculated as the difference between the lowest and the largest detected means) and an α of .05 by 2-sided test (see eMethods for details).

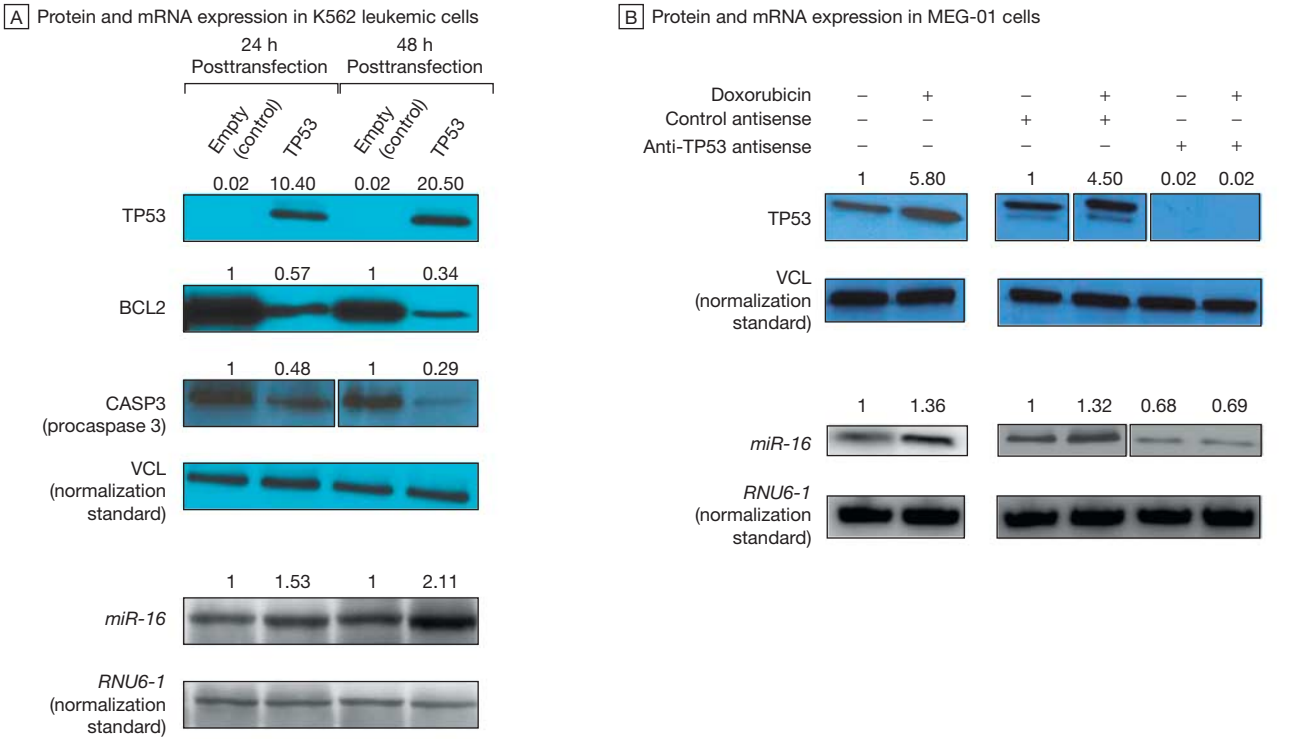
RESULTS

13q-17p Molecular Link in Patients With B-CLL

The clinical characteristics of the 188 consecutive patients with untreated B-CLL are shown in the Table. No departures from Hardy-Weinberg equilibrium were detected for any of the 3 chromosomal abnormalities investigated. The 13q deletion was the chromosomal abnormality that occurred most often in patients with CLL and was frequently the only cytogenetic abnormality in these patients, whereas the other 2 most common chromosomal abnormalities in CLL (11q deletion and 17p deletion) were often associated with the 13q deletion, but they rarely occurred together in 1 clone.

Patients (n=22) with CLLs with a homozygous 13q deletion (13q–/–) had significantly lower expression levels of *miR-15a* (mean fold induction, 0.04 [95% CI, 0.035-0.045]; *P*=.03) and *miR-16* (mean fold induction, 0.18 [95% CI, 0.16-0.20]; *P*=.01) than patients (n=28) with CLLs with normal cytogenetic profiles (defined as no abnormality detected by FISH) (set as fold induction, 1 [95% CI, 0.94-1.05] for both microRNAs). Conversely, patients with 13q–/– CLLs had significantly higher *TP53* expression levels than patients with CLLs with normal cytogenetic profiles, both at the mRNA level (mean fold induction, 4.83 [95% CI, 4.36-5.30] vs 1 [95% CI, 0.91-1.09]; *P*=.03) and at the protein level (mean fold induction, 2.06 [95% CI, 1.98-2.14] vs 1 [95% CI, 0.96-1.04]; *P*=.04) (eFigure 2). Also, patients with CLLs with a heterozygous 13q deletion (13q+/–) had significantly lower expression levels of *miR-15a* and *miR-16* than patients with CLLs with normal cytogenetic profiles (mean fold induction, 0.48 [95% CI, 0.46-0.50]; *P*=.03 and 0.64 [95% CI, 0.62-0.66]; *P*=.04 vs 1 [95% CI, 0.97-1.03], respectively) (eFigure 3A). Patients with 13q+/– CLLs had significantly higher *TP53* expression levels

Figure 4. Transactivation of MicroRNA *miR-16* Affecting Expression of *miR-16* Targets



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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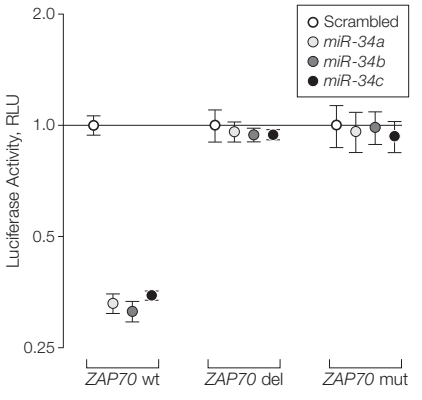
Figure 5. Targeting of *ZAP70* by *miR-34a, miR-34b*, and *miR-34c*



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.

than those with CLLs with normal cytogenetic profiles both at the mRNA level (mean fold induction, 2.32 [95% CI, 2.23-2.41]; $P = .03$ vs 1 [95% CI, 0.97-1.03]) and at the protein level (mean fold induction, 1.48 [95% CI, 1.45-1.51]; $P = .04$ vs 1 [95% CI, 0.97-1.03]), although TP53 expression in patients with 13q+/– CLLs was not as high as that in patients with 13q–/– CLLs (eFigure 3A and B).

A slight inverse correlation was found between expression of *miR-15a/miR-16* and *TP53* mRNA ($r = –0.31$; $P < .04$) in patients with 13q–/– CLLs compared with patients with CLLs with normal cytogenetic profiles. In MEG-01 cells, TP53 protein expression was highly reduced when the *miR-15a/miR-16-1* cluster was overexpressed (eFigure 4). Overexpression of each microRNA of the cluster was associated with reduced TP53 expression (52% for *miR-15a* and 62% for *miR-16*) compared with a scrambled oligonucleotide control, and the combination of both *miR-15a* and *miR-16* almost completely repressed TP53 expression (TP53 protein expression was reduced by 82%) (FIGURE 1A). No TP53 silencing effect was observed with the antisense oligonucleotides. Both *miR-15a* and *miR-16* were found to target BCL2 (Figure 1A), which is consistent with our previous findings.[12] In MEG-01 cells stably expressing *miR-16*, TP53 expression was reduced, as were the protein levels of its downstream effectors CDKN1A, BBC3, and BCL2 (eFigure 5). Additionally, cleavage of procaspase 3 was observed in MEG-01 cells stably expressing *miR-15a* or *miR-16-1*, confirming that the *miR-15a/miR-16-1* cluster has a caspase-dependent proapoptotic role in this cell line (eFigure 5). No effect on CDKN1A and BBC3 was observed in TP53-negative H1299 cells (eFigure 6), which suggests that the effects of *miR-15a* and *miR-16* on CDKN1A and BBC3 are mediated by their effects on TP53. Conversely, the targeting effect of *miR-15a* and *miR-16* on BCL2 persisted also in TP53-null cells (eFigure 6). These results were confirmed in primary B-CLL cells collected from 3 patients with 13q–/– CLL. When *miR-15a* and *miR-16* were overexpressed in these primary B-CLL cells, the expression of *TP53*, *CDKN1A*, *BBC3*, and *BCL2* was reduced both at the protein and mRNA level (Figure 1B and eFigure 7 [left panels]).

A binding site for both *miR-15a* and *miR-16* inside the 3′-UTR (untranslated region) of *TP53* was identified using the sequencer software (eFigure 8). A luciferase reporter assay showed that both *miR-15a* and *miR-16* directly target the identified *TP53* binding site and significantly reduced the luciferase reporter activity compared with a scrambled oligonucleotide-negative control (mean luciferase activity, 0.68 relative light units [RLU] [95% CI, 0.63-0.73]; $P = .02$ for *miR-15a* and 0.62 RLU [95% CI, 0.59-0.65]; $P = .02$ for *miR-16* vs 1 [95% CI, 0.94-1.06] for scrambled). This effect was completely abolished when the binding site was either deleted or mutated (FIGURE 2A). Lastly, by using a *miR-15a/miR-16-1* doxycycline-inducible HeLa cell line when *miR-16* was down-regulated in presence of doxycycline, the expression of *TP53* mRNA was significantly increased (mean fold induction, 6.39 [95% CI, 5.46-7.32] vs 1 [95% CI, 0.46-1.54]; $P = .01$) (Figure 2B).

17p-13q Molecular Interaction in Patients With B-CLL

Several TP53 binding sites were found upstream of the 2 homologous *miR-15/miR-16* loci located on chromosome 13 (*miR-15a/miR-16-1*) and on chromosome 3 (microRNA 15b [*miR-15b*] [GenBank 406949]/microRNA 16-2 [*miR-16-2*] [GenBank 406951]), which was also analyzed because it encodes for a similar cluster of genes (eFigure 9). Chromatin immunoprecipitation analysis revealed that TP53 directly binds to its predicted binding sites on both chromosome 13 and chromosome 3, both in cell lines and in primary CLLs with normal cytogenetic profiles (eFigure 10 and eFigure 11). A luciferase reporter assay showed that TP53 significantly increased the luciferase reporter activity of all the binding site–containing vectors (FIGURE 3A). In MEG-01 cells, TP53 transactivation of the *miR-15/miR-16* cluster was also confirmed by qRT-PCR (Figure 3B); this transactivating effect also occurred in TP53-mutated K562 cells, in which it was associated with a reduction of procaspase 3 protein levels (FIGURE 4A). Similarly, doxorubicin-mediated TP53 activation in MEG-01 cells increased the expression of *miR-16*, an effect that was abolished when TP53 was silenced by an anti-TP53 oligonucleotide (Figure 4B). Although the effect of TP53 on the *miR-15/miR-16* cluster was relatively mild (highest fold induction of 2.11 after 48 hours), it still had functional consequences and was associated with 66% reduction of BCL2 protein levels after 48 hours.

17p-11q-*ZAP70* Molecular Link in Patients With B-CLL

Chromatin immunoprecipitation analysis revealed that TP53 binds

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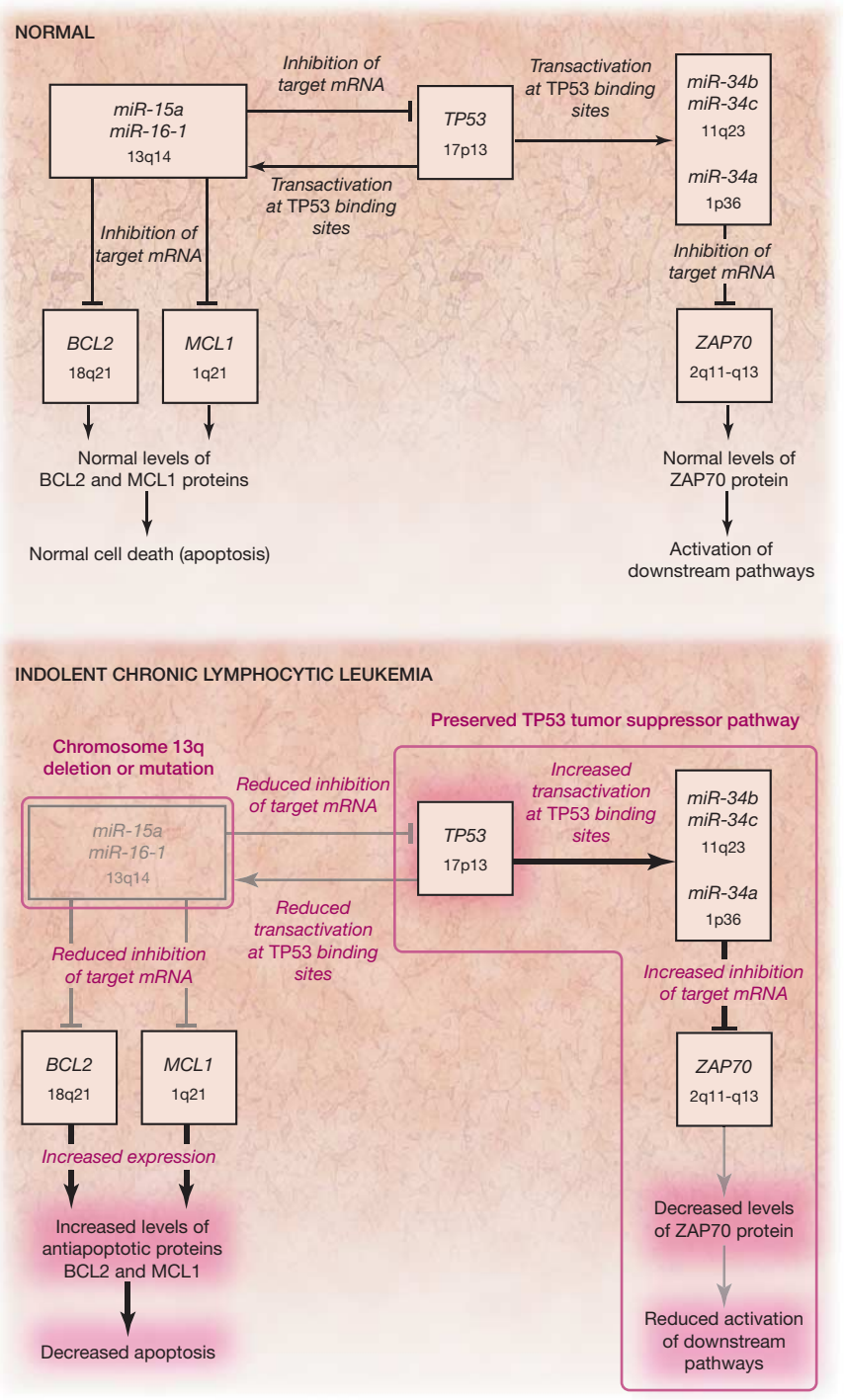
directly to a pre–*miR-34b/miR-34c* TP53 binding site on chromosome 11 (eFigure 10 and eFigure 12). A luciferase reporter assay showed a statistically significant transactivating effect of TP53 on the *miR-34b/miR-34c* binding site (Figure 3A), which was confirmed in TP53-transfected MEG-01 cells, which 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, 2.02 [95% CI, 1.98-2.06] vs 1 [95% CI, 0.97-1.03]; $P=.01$) and at the protein 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

Figure 6. MicroRNA/TP53 Pathogenetic Model for Human CLL



A novel pathogenetic model for chronic lymphocytic leukemia (CLL) showing a pathway of microRNAs and protein coding genes that are involved in the development of CLL. The microRNA 15a (*miR-15a*)/microRNA 16-1 (*miR-16-1*) cluster, the microRNA 34b (*miR-34b*)/microRNA 34c (*miR-34c*) cluster, and the genes tumor protein p53 (*TP53*), B-cell CLL/lymphoma 2 (*BCL2*), myeloid cell leukemia sequence 1 (BCL2-related) (*MCL1*), and zeta-chain (TCR)–associated protein kinase 70kDa (*ZAP70*) are the main partners in this model. mRNA indicates messenger RNA.

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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* vs 1 [95% CI, 0.94-1.06] for scrambled). This effect was abolished when the predicted site on *ZAP70* was either deleted or mutated (FIGURE 5). In K562 cells as well as primary B-CLL lymphocytes, increased levels of *ZAP70* mRNA were observed after the *miR-34* family was silenced (eFigure 13C and D).

Reexpression of *miR-15a* and *miR-16* in primary B-CLL cells from patients with 13q–/– CLL was associated with a reduction of TP53; reduction of *miR-34a, miR-34b, and miR-34c*; and increased expression of ZAP70 protein levels (eFigure 7 [right panels] and Figure 1B).

COMMENT

In this study, we identified a microRNA/protein functional circuitry that likely underlies the pathogenesis and natural history of a major subset of human CLL. This pathway is perturbed at different levels by distinct CLL chromosomal abnormalities, which could explain the occurrence of the same disease but with distinct clinical features. This novel pathogenetic model for CLL is summarized in FIGURE 6.

In this model, *TP53* (located on chromosome 17p) represents the molecular link between the *miR-15a/miR-16-1* (located on chromosome 13q) and *miR-34b/miR-34c* (located on chromosome 11q) clusters. In fact, the tumor suppressor protein TP53 is directly regulated by *miR-15a/miR-16-1*. The loss of *miR-15a/miR-16-1* expression, represented by CLLs with 13q deletions, not only shifts the balance toward higher levels of the antiapoptotic proteins BCL2 and myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), as we have previously demonstrated,[12,16] but also toward higher levels of the tumor suppressor protein TP53. Consequently, in patients with CLLs with 13q deletions, while the number of apoptotic cells may decrease because of the increased levels of antiapoptotic proteins, the TP53 tumor suppressor pathway remains intact, thus keeping the increase in tumor burden relatively low (eComments).

This novel finding explains how 13q deletions are associated with the indolent form of CLL, as first identified by Döhner et al.[4] Moreover, increased TP53 levels, as found in patients with CLLs with 13q deletions, are associated with transactivation of *miR-34b/miR-34c* and reduced levels of ZAP70, a tyrosine kinase relevant in the initial step of T-cell receptor–mediated signal transduction.[17] Low expression levels of ZAP70 have been found to be positively correlated with survival in patients with CLL,[18] 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 TP53, *miR-34a*, *miR-34b*, and *miR-34c* and increased protein levels of ZAP70. 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 TP53. 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 H1299, A549, and HeLa cells), which suggests that the proposed microRNA-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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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)

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