Title

TCL1 targeting miR-3676 is codeleted with tumor protein p53 in chronic lymphocytic leukemia

Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(7)

ISSN

0027-8424

Authors

Balatti, Veronica
Rizzotto, Lara
Miller, Cecelia
et al.

Publication Date

2015-02-17

DOI

10.1073/pnas.1500010112

Peer reviewed

TCL1 targeting *miR-3676* is codeleted with tumor protein p53 in chronic lymphocytic leukemia

Veronica Balatti[a], Lara Rizzotto[a], Cecelia Miller[b], Alexey Palamarchuk[a], Paolo Fadda[a], Rosantony Pandolfo[a,c], Laura Z. Rassenti[d], Erin Hertlein[b], Amy S. Ruppert[b], Arletta Lozanski[b], Gerard Lozanski[b], Thomas J. Kipps[d], John C. Byrd[b], Carlo M. Croce[a,1], and Yuri Pekarsky[a,1]

[a]Department of Molecular Virology, Immunology and Medical Genetics, and [b]Division of Hematology, The Ohio State University Comprehensive Cancer Center, Columbus, OH 43210; [c]Department of Health Sciences, University "Magna Grecia" of Catanzaro, Catanzaro 88100, Italy; and [d]Department of Medicine, University of California, San Diego, La Jolla, CA 92093

Contributed by Carlo M. Croce, January 6, 2015 (sent for review November 1, 2014; reviewed by Kanti R. Rai and Frank Slack)

B-cell chronic lymphocytic leukemia (CLL) is the most common human leukemia and dysregulation of the T-cell leukemia/lymphoma 1 (*TCL1*) oncogene is a contributing event in the pathogenesis of the aggressive form of this disease based on transgenic mouse studies. To determine a role of microRNAs on the pathogenesis of the aggressive form of CLL we studied regulation of *TCL1* expression in CLL by microRNAs. We identified *miR-3676* as a regulator of *TCL1* expression. We demonstrated that *miR-3676* targets three consecutive 28-bp repeats within 3′UTR of *TCL1* and showed that *miR-3676* is a powerful inhibitor of *TCL1*. We further showed that *miR-3676* expression is significantly down-regulated in four groups of CLL carrying the 11q deletions, 13q deletions, 17p deletions, or a normal karyotype compared with normal CD19$^+$ cord blood and peripheral blood B cells. In addition, the sequencing of 539 CLL samples revealed five germ-line mutations in six samples (1%) in *miR-3676*. Two of these mutations were loss-of-function mutations. Because *miR-3676* is located at 17p13, only 500-kb centromeric of tumor protein p53 (*Tp53*), and is codeleted with *Tp53*, we propose that loss of *miR-3676* causes high levels of *TCL1* expression contributing to CLL progression.

miR-3676 | CLL | 17p deletions

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia (1). Unfavorable prognosis associates with the expression of unmutated immunoglobulin variable genes (*IGVH*) and high levels of 70 kD zeta-associated protein (ZAP-70). CLLs can be subgrouped into aggressive and indolent cases and chromosomal aberrations are present in more than 80% of patients (1–3). Cytogenetic abnormalities are also a prognostic parameter (3). The most common chromosomal abnormalities detectable by cytogenetics include deletion at 13q (50%), 11q (18%), trisomy 12 (15–18%), and 17p (8%) (2, 3). Prognostic categories have shown poor survival in patients harboring 17p deletion, 11q deletion, or trisomy 12, and better survival for patients with normal karyotype and 13q deletion as the sole abnormality (3).

We have shown that loss of *miR-15a/16-1* is the most common genetic alteration in CLL (70–80%) (4). This alteration is very common in indolent CLLs and in indolent CLLs that progress to the aggressive form (4, 5). Previously, we have observed loss of *miR-181* and *miR*-29, which negatively regulate T-cell leukemia/lymphoma 1 (*TCL1*), during the progression of CLL (6, 7).

Activation of the *TCL1* gene is arguably one of most studied events in the pathogenesis of aggressive CLL (8). We and others have found that *TCL1* overexpression occurs in a number of B-cell malignancies, including CLL (8–10). To validate that overexpression of *TCL1* is oncogenic in B-cells, we have created a Eμ-*TCL1* transgenic mouse model overexpressing *TCL1* (11, 12). All of these transgenic mice developed the aggressive form of CLL at the average age of 12 mo with 100% penetrance (11, 12), indicating that deregulation of *TCL1* is critically important in the pathogenesis of the aggressive form of CLL. Numerous publications of this CLL model have confirmed our findings (13–17). Previously we reported that Tcl1 overexpression contributes to CLL pathogenesis by coactivating Akt, and inhibiting AP-1 and de novo DNA methylation by inhibiting of the de novo DNA methyltransferases (18–21). The mechanisms responsible for overexpression of Tcl1 in CLL are not entirely clear. For this reason, we studied the regulation of *TCL1* expression by microRNAs.

Results

The goal of this study was to identify microRNAs targeting *TCL1* that could play a role in the development and progression of CLL by modulating *TCL1* expression. Because the 3′UTR of *TCL1* contains three consecutive 28-bp repeats (NM_021966.2, nucleotides 935–962, 964–991, and 993–1020) (Fig. 1*A*), we investigated whether each of these repeats could be targeted by microRNAs. Using TargetScan (www.targetscan.org) software we identified four microRNAs—*miR-885*, *miR-1282*, *miR-3676*, and *miR-4514*—predicted to target each of the three consecutive 28-bp repeats. Previously, we reported that Tcl1 is expressed at low levels in indolent CLLs, whereas aggressive CLLs generally overexpress Tcl1 (6). To investigate whether these four microRNAs play a role in the pathogenesis of CLL, we chose four samples from indolent CLL patients with the 13q deletion, and four samples from aggressive CLL patients with the 11q deletion. Our goal was to find microRNAs showing higher expression in the indolent samples compared with the aggressive samples (Fig. 1*B* and Table S1). This approach was based on our previous observation that the most

Significance

B-cell chronic lymphocytic leukemia (CLL) is the most common adult leukemia. We previously found that dysregulation of the T-cell leukemia/lymphoma 1 (*TCL1*) oncogene is a critical contributing event in the pathogenesis of this disease. In this study we investigated molecular causes of *TCL1* overexpression in CLL. We identified *miR-3676* as a powerful regulator of *TCL1* expression. We found that *miR-3676* is down-regulated on all groups of CLLs and mutated in 1% of CLLs. Interestingly, *miR-3676* is located at 17p13, only 500-kb centromeric of tumor protein p53 (*Tp53*), and is codeleted with *Tp53* in 17p-deleted CLL. Loss of *miR-3676* causes high levels of *TCL1* expression contributing to CLL progression. Thus, this study uncovers a major mechanism in the pathogenesis of CLL.

Author contributions: J.C.B., C.M.C., and Y.P. designed research; V.B., L.R., C.M., A.P., P.F., R.P., E.H., A.S.R., A.L., and G.L. performed research; L.Z.R., T.J.K., and J.C.B. contributed new reagents/analytic tools; V.B. analyzed data; and Y.P. wrote the paper.

Reviewers: K.R.R., The Feinstein Institute for Medical Research; and F.S., Harvard University.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. Email: carlo.croce@osumc.edu or pekarsky.yuri@osumc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500010112/-/DCSupplemental.

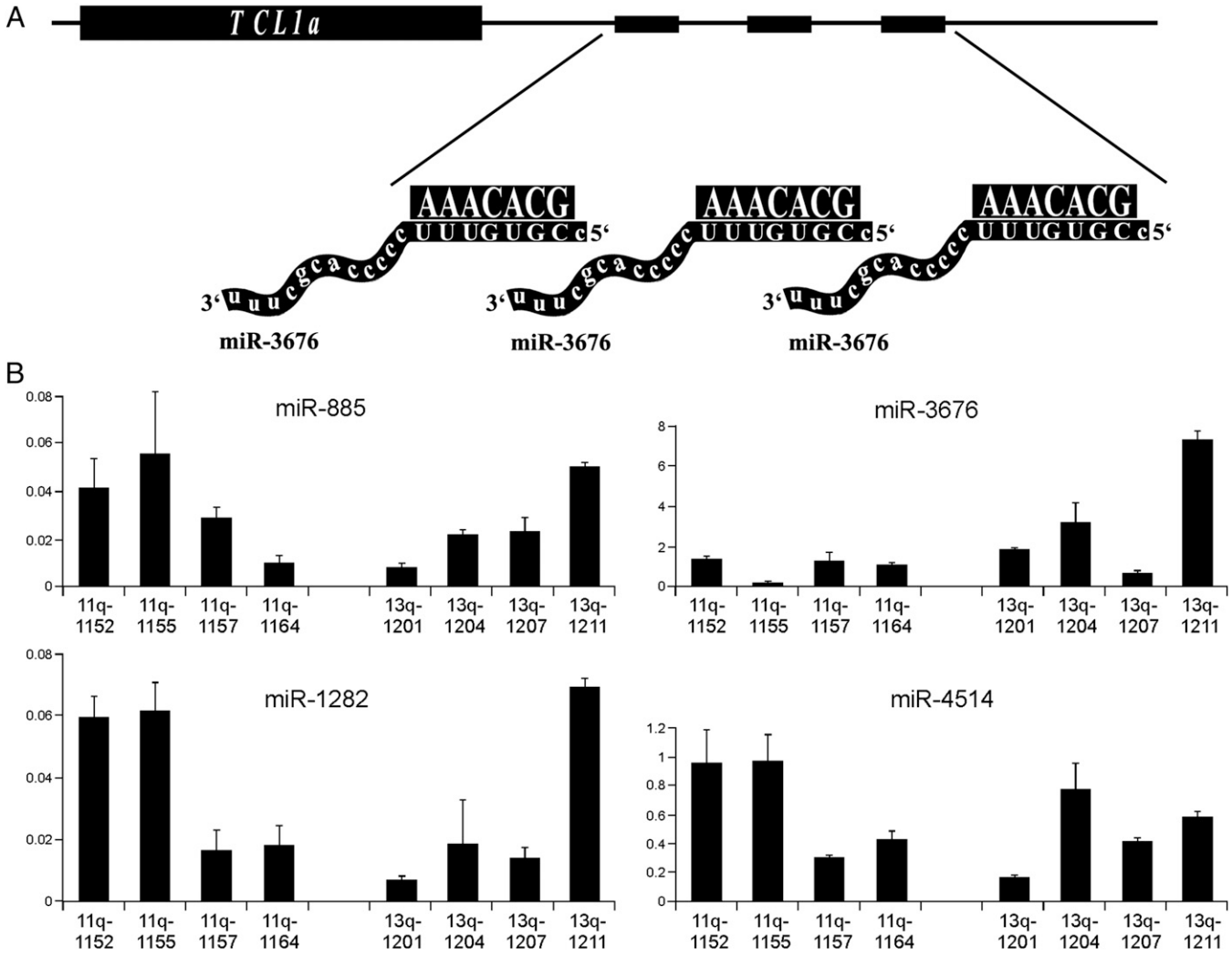

Fig. 1. MicroRNAs targeting three consecutive repeats within *TCL1* 3′UTR. (*A*) Three repeats within *TCL1* 3′UTR. (*B*) Expression of *miR-885, miR-1282, miR-3676*, and *miR-4514* in 11q$^-$ and 13q$^-$ CLL. For the *y* axes, relative expression of microRNAs normalized versus U6 control.

aggressive 11q deleted CLLs show highest Tcl1 expression levels (6). Fig. 1*B* shows expression of *miR-885* (average CT was ∼36), *miR-1282* (average CT was ∼36), *miR-3676* (average CT was ∼30), and *miR-4514* (average CT was ∼31.5) in these eight CLL samples measured by real-time RT-PCR. Thus, of the four microRNAs tested, *miR-885* and *miR-1282* were practically not expressed, and only expression of *miR-3676* was down-regulated in the 11q-deleted aggressive samples compared with the 13q-deleted indolent samples (Fig. 1*B*). Thus, we decided to further investigate the *miR-3676* (we also considered *miR-4514*, see below).

We first identified the chromosomal location of *miR-3676* using University of California, Santa Cruz Genome Browser (www.genome.ucsc.edu). *MiR-3676* was first identified from deep-sequencing data as 1 of the 30 most abundant microRNAs in breast cancer cells (it was referred as *hsa-miR-B13f*) (22). Very interestingly, *miR-3676* is located at 17p13, only 500-kb centromeric of *TP53*, within the 17p-deleted region in CLL (2), and partially overlaps with tRNA-Thr (Fig. 2*A*). Thus, *miR-3676* is codeleted with *TP53* in CLLs with 17p deletions. We then studied the expression levels of *miR-3676* in CLL compared with CD19$^+$ B-cells isolated from peripheral blood mononuclear cells and cord blood using a larger set of samples subgrouped by their karyotype. The set included 28 samples with 11q deletions, 9 samples with 17p deletions, 11 samples with 13q deletions, and 15 samples with a normal karyotype for a total of 63 CLLs (Table S1). The real-time RT-PCR data are shown in Fig. 2 *B–D*. Interestingly, *miR-3676* expression was statistically significantly down-regulated in CLLs of all karyotypes (2- to 5-fold compared with CD19$^+$ B-cells from peripheral blood mononuclear cells, and 9- to 20-fold compared with CD19$^+$ B-cells from cord blood) (Fig. 2*D*). As we expected, the lowest expression levels of *miR-3676* were observed in the 17p-deleted CLLs (these samples contain only one allele of *miR-3676*) (Fig. 2*D*). *miR-3676* expression in 17p-deleted samples was 1.7-fold lower vs. 11q-deleted samples (P = 0.15), 2.2-fold lower vs. 13q-deleted samples (P = 0.059), and 2.6-fold lower versus normal karyotype samples (P = 0.01) (Fig. 2*D*). On the other hand, *miR-3676* expression in 13q-deleted samples was only 1.3-fold lower vs. 11q-deleted samples (Fig. 2*D*). This difference was smaller than the one in Fig. 1*B*, likely because the most aggressive 11q samples were chosen for the initial experiments (average ZAP-70 = 57.4% and VH = 100% in Fig. 1*B* compared with ZAP-70 = 24.0% and VH = 99.4% in the bigger set of samples in Fig. 2*D*) (Table S1). Similarly, for four 13q-deleted samples in Fig. 1*B*, the average ZAP-70 was 1.1% and in the larger cohort average Zap-70 was 3.9% (Table S1). In contrast, no down-regulation of *miR-4514* expression was observed in CLL samples vs. CD19$^+$ cord blood B cells (Fig. S1). Differential down-regulation of *miR-3676* in CLL

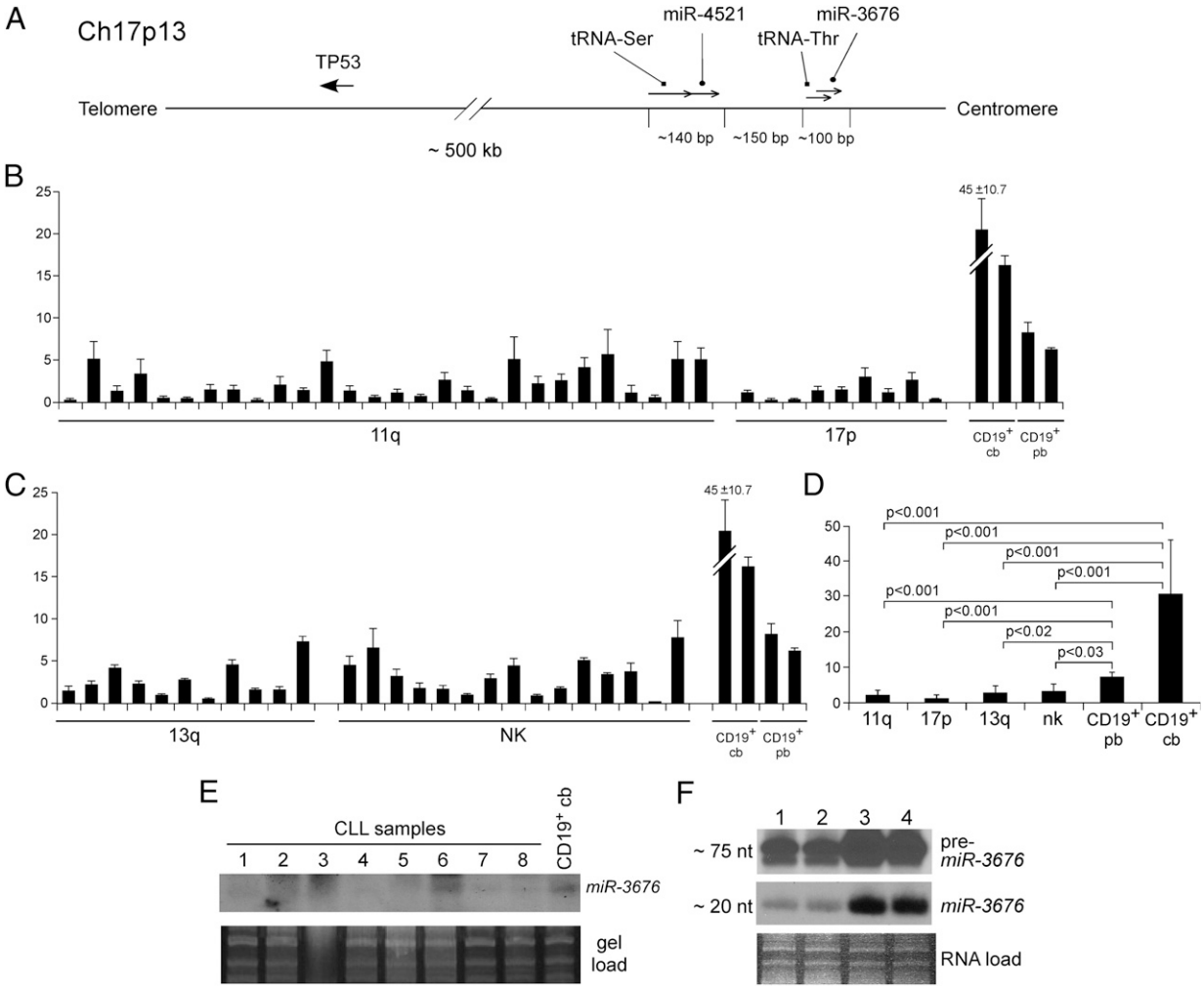

Fig. 2. *miR-3676* expression is down-regulated in CLL. (*A*) Genomic location of *miR-3676*. (*B*) *miR-3676* expression is down-regulated in $11q^-$ and $17p^-$ CLL. (*C*) *miR-3676* expression is down-regulated in $13q^-$ and normal karyotype CLL. (*D*) Summary of *miR-3676* expression in CLL and normal controls. (*E*) Northern blot analysis of *miR-3676* expression in CLL samples and $CD19^+$ cord blood cells. (*F*) Northern blot of nontransfected HEK-293 cells (lane 1) and HEK-293 cells transfected with empty vector, pCDH-3676/4521 or pCDH-3676 (lanes 2–4, respectively).

was also confirmed by Northern blot analysis (Fig. 2*E*); of the eight CLL samples that were loaded on this gel, only sample #6 had a band comparable with the band observed for $CD19^+$ B-cells (Fig. 2*E*). Because *miR-3676* partially overlaps with tRNA-Thr (Fig. 2*A*), we verified that this genomic fragment actually expresses a microRNA. We cloned a fragment containing *miR-4521*, *miR-3676*, and two corresponding tRNAs, (pCDH-3676/4521) and a fragment containing only *miR-3676* and tRNA-Thr (pCDH-3676) (Fig. 2*A*) into a pCDH expression vector. Fig. 2*F* shows a Northern blot of HEK-293 cells transfected with empty vector, pCDH-3676/4521, or pCDH-3676 (lanes 2–4, respectively). As shown in Fig. 2*F*, HEK-293 cells express both, endogenous

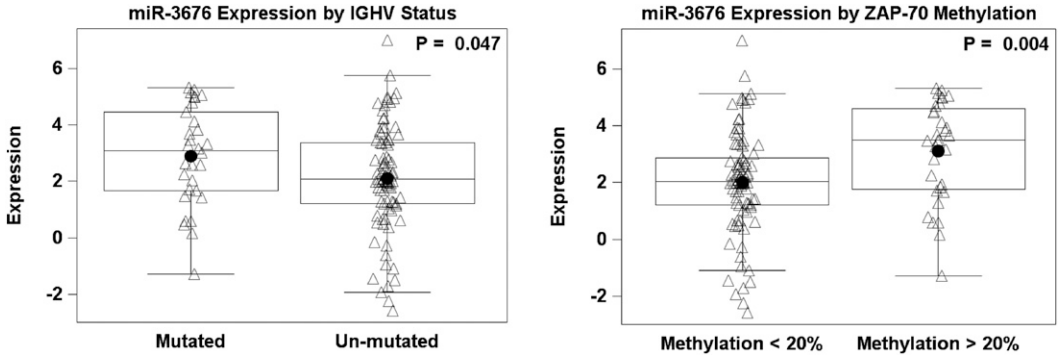

Fig. 3. Association between *miR-3676* expression IGVH status and ZAP-70 methylation.

(lanes 1 and 2) and exogenous (lanes 3 and 4) *miR-3676*. The full Northern blot (two different exposures) of Fig. 2*F* is shown in Fig. S2, demonstrating that *miR-3676* is ~20 nucleotides in size and its size is identical to commercially bought *miR-3676*.

We have recently identified ZAP-70 promoter methylation as a strong predictor of patient outcome (21, 23), 117 additional previously untreated CLL patients for whom *ZAP-70* methylation, ZAP-70 protein expression, IGVH mutational analysis, and CD38 expression had been assessed were examined for expression of *miR-3676*. Whereas significant associations with ZAP-70 or CD38 protein levels by flow cytometry were not attained, expression of *miR-3676* was 2.15-fold higher, on average, in the high *ZAP-70* methylated patients versus those who had lower levels of methylation [95% confidence interval (CI): 1.29–3.60; $P = 0.004$] and 1.72-fold higher, on average, in the mutated IGVH patients versus those with unmutated disease (95% CI: 1.01–2.94; $P = 0.047$) (Fig. 3).

Because *TCL1* is a predicted target of *miR-3676*, we proceeded to determine whether this microRNA indeed targets Tcl1 expression. We cloned full-length human *TCL1* cDNA including 5′ and 3′UTRs into CMV mammalian expression vector and investigated whether *miR-3676* affects Tcl1 protein expression levels. We cotransfected this construct with *miR-3676* and scrambled control into HEK-293 cells in two different concentrations (Fig. 4*A*). These experiments revealed that coexpression of *TCL1* with *miR-3676* completely abolished Tcl1 expression, even when a lower concentration of *miR-3676* was used (Fig. 4*A*). These results are quite striking because similar experiments with other microRNAs usually result just in a decrease of the expression of target proteins (6, 24). To confirm our findings we inserted a fragment of the *TCL1* 3′UTR containing all three 28-bp repeats downstream of the luciferase ORF in the pGL3 vector, as previously described (25). We then mutated two of three or all three *miR-3676* target sequences in this construct. HEK-293 cells were cotransfected with *miR-3676* or scrambled negative control, as indicated, and a pGL3 construct containing three, one, or zero sites homologous to *miR-3676* (Fig. 4*B*). Although the presence of three or one *miR-3676* target sequences in these experiments significantly inhibited luciferase expression, there was no difference in luciferase expression when all three sites were mutated (Fig. 4*B*). Thus, we concluded that *miR-3676* drastically inhibits *TCL1* expression at the mRNA and protein levels. These data are quite interesting because they indicate that multiple target sites of microRNAs at the 3′UTR of the target gene can dramatically suppress its expression. To confirm that *miR-3676* targets *TCL1* in CLL, we chose a set of 49 17p-deleted samples and determined *miR-3676* expression in this set. These results are shown in Fig. S3 and Tables S2 and S3. To prove that Tcl1 expression is decreased in samples with higher *miR-3676* expression, we carried out Western blot analysis using six samples with the highest *miR-3676* expression and six patients with the lowest *miR-3676* expression. Fig. 4*C* shows that Tcl1 expression levels were significantly lower in samples with high *miR-3676* expression (~2.5-fold, $P = 0.03$).

To determine if *miR-3676* is mutated in CLL, we sequenced 545 samples from CLL and 146 DNA sampled from healthy individuals. We found five different mutations in six CLL samples (~1%) within the *miR-3676*, including two mutations within the mature microRNA (Fig. 5*A* and Table S4). In addition, we found a mutation in one CLL sample 41 bp downstream of *miR-3676* (Fig. 5*A* and Table S4), and one mutation in one normal sample 3 bp downstream of *miR-3676* (Chr. 17, 8090580, C to T). All mutations found were heterozygous, and all mutations found in CLL were germ-line mutations because they were also present in T cells sorted from the same samples. Because two of these mutations were in the mature *miR-3676* [C to T (M9) and ins C (M4)] (Fig. 5*A*), we investigated whether these mutations are loss-of-function mutations. We cotransfected HEK-293 cells with *TCL1* construct used in Fig. 4*A* and pCDH-3676/4521 pCDH-4521 (C), pCDH-3676/4521 WT (WT), pCDH-3676/4521 M4 (M4), or pCDH-3676/4521 (M9) (Fig. 5*B*; experiments were carried out in duplicates). Although expression of WT *miR-3676* significantly inhibited Tcl1 expression, both M4 and M9 mutants did not show any inhibition of Tcl1 (Fig. 5*B*). We thus concluded that these two mutants are loss-of-function mutants.

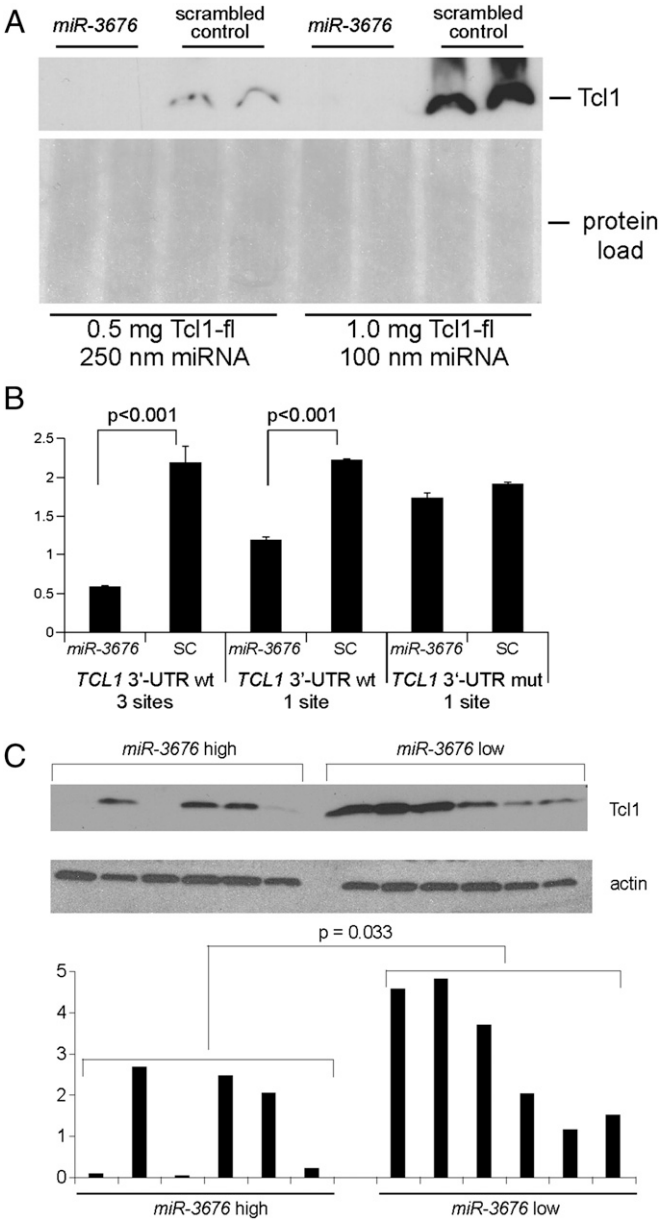

Fig. 4. Tcl1 expression is targeted by *miR-3676*. (*A*) Western blot analysis of *TCL1* cotransfected with *miR-3676* or scrambled control. Transfections were carried out with indicated constructs and oligos. (*B*) *miR-3676* targets Tcl1 expression in luciferase assays. HEK-293 cells were cotransfected with *miR-3676* or scrambled negative control, as indicated, and pGL3 construct containing three (*TCL1* 3′UTR WT, three sites), one (*TCL1* 3′-UTR WT, one site), or zero sites (*TCL1* 3′UTR mut, one site) homologous to *miR-3676*. For the *y* axes, relative luciferase expression normalized versus *Renilla* expression. Firefly and *Renilla* luciferase activities were assayed with the dual luciferase assay system (Promega) and firefly luciferase activity was normalized to *Renilla* luciferase activity, as suggested by the manufacturer. (*C*) Tcl1 expression in 17p deleted CLL samples with high and and low miR-3676 expression. (*Upper* and *Middle*) Western blot using anti-Tcl1 and anti-actin antibodies. (*Lower*) Normalized expression of Tcl1 versus actin.

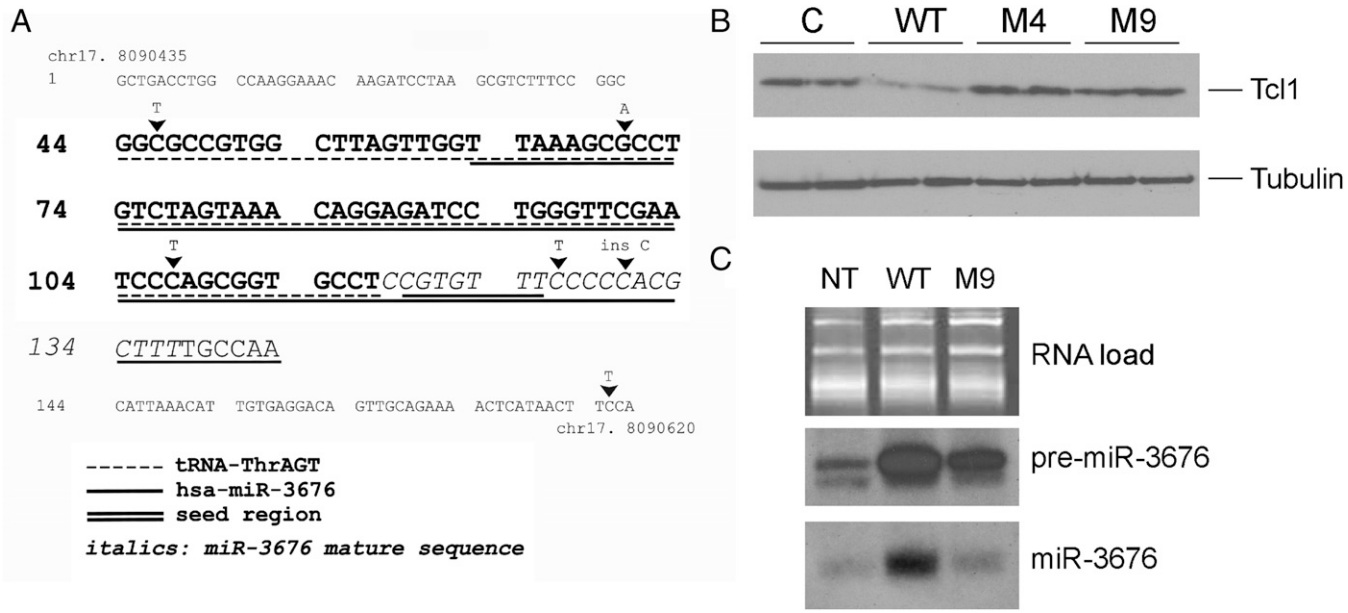

Fig. 5. (*A*) *miR-3676* mutations in CLL. (*B*) M4 and M9 mutations are loss of function mutations. (*C*) M9 mutation inhibits transcription of *miR-3676*. NT, untransfected control.

Most microRNAs are transcribed by RNA polymerase II, but recent studies showed increasing number of cases in which RNA polymerase III is involved (26, 27). Because *miR-3676* partially overlaps with tRNA and tRNAs are normally transcribed by RNA polymerase III (28), it is possible that RNA polymerase III is involved in *miR-3676* transcription. The stretch of four or more T residues is a transcription termination stop for RNA polymerase III (29). We noticed that the M9 mutation creates the stretch of four T residues (it creates TTTT sequence from TTTC) (Fig. 5*A*). If this termination signal is used, that would eliminate expression of *miR-3676* (Fig. 5*A*). To check this possibility, we carried out Northern blot of the exogenous *miR-3676* and the M9 mutant (Fig. 5*C*). HEK-293 cells were transfected with pCDH-3676/4521 WT or pCDH-3676/4521 M9. The probe used in this experiment contained 50% of labeled oligonucleotide complementary to WT *miR-3676* and 50% of that complimentary to mutant M9. Indeed, the M9 mutation significantly inhibited expression of *miR-3676* (Fig. 5*C*), confirming that this mutation is a loss-of-function mutation.

Discussion

Deletions of the short arm of chromosome 17 are common in the treatment of refractory CLLs and observed in ∼8% of all CLL patients (2, 30). The prominent tumor-suppressor gene *TP53* is located within this deleted region and is considered the main target of these deletions (30, 31). *TP53* is also mutated in ∼10% of CLL patients at diagnosis or before first therapy and these mutations are associated with poor prognosis (30–32). No other candidate gene target at 17p is currently under investigation. On the other hand, these deletions are several megabases long and contain a number of genes (33). *miR-3676* is located ∼500-kb centromeric of *Tp53* and is deleted in all cases where *TP53* is also deleted. Notably, *miR-3676* was recently removed from miRbase database (www.mirbase.org) because of its overlapping with tRNA-Thr (Fig. 5*A*). However, data presented here (specifically in Figs. 2*F* and 5*C* and Fig. S2), and the fact that *miR-3676* was previously identified by sequencing data as one of the 30 most abundant microRNAs in breast cancer cells (22), provide unquestionable support for this microRNA. Because mature *miR-3676* starts exactly at the end tRNA-Thr (Fig. 5*A*) and ends at a transcription termination stop for RNA polymerase III, we cannot exclude the possibility that *miR-3676* represents a member or a new class of small RNAs, tRNA-derived small RNAs (tsRNAs) generated during tRNA processing (34, 35). Such tsRNA would be a single stranded RNA molecule identical to the mature *miR-3676*.

In this report we demonstrate that *miR-3676* is down-regulated in the different cytogenetic groups of CLL, mutated in ∼1% of CLL, and deleted with the loss of 17p. A recent report assessed effects of *TP53* inactivation in *TCL1* mouse model of CLL (36). This paper demonstrates that *TP53* deletion strongly cooperates with Tcl1 overexpression in CLL pathogenesis in every characteristic measured (survival, spleen size, white blood count, and so forth). This finding fits well with codeletion of *Tp53* and *miR-3676* in 17p-deleted CLLs: *TP53* deletion leads to the loss of p53 function (as other allele is often mutated), *miR-3676* deletion leads to Tcl1 overexpression, creating cooperation of both events. B-cell receptor signaling is one of critical pathways in CLL pathogenesis (37). A recent study showed that ibrutinib, an inhibitor of this pathway, slowed CLL development in *TCL1* CLL mouse model (38). It is of interest to investigate if this drug could work in *TP53* deleted *TCL1* transgenics because these mice recapitulate 17p-deleted CLLs. This drug was already approved to treat 17p-deleted human CLLs and it could also be an attractive agent to use in patients with monoclonal B-cell lymphocytosis, a known precursor of clinical CLL to determine if it can prevent the development of CLL.

The fact that *miR-3676* is down-regulated not only in 17p-deleted CLLs but in all CLL groups, suggests that mechanisms other than deletions and mutations inhibiting *miR-3676* expression exist. It is of importance to investigate and determine what these mechanisms are. In this respect, *miR-3676* is similar to the tumor-suppressor cluster *miR-15/16* located at 13q. Like *miR-3676*, *miR-15/16* is inactivated in CLL by several mechanisms: deletions (4), mutations (39), and processing defects (40). We demonstrate the loss of *miR-3676* is also associated with low *ZAP-70* promoter methylation and IGVH unmutated disease. This finding seems logical because Tcl1 overexpression (and loss of *miR-3676* is a contributing factor to that) is a hallmark of the

aggressive form of CLL characterized by unmutated IGVH and higher *ZAP-70* expression.

We propose that the loss of microRNAs targeting the 3′UTR of *TCL1*, particularly of *miR-3676*, results in the dysregulation of *TCL1* to levels comparable to those observed in Eμ-*TCL1* transgenic mice that develop the aggressive form of CLL (11). Further exploration of *miR-3676*'s role predicting CLL predisposition, progression, and influence on treatment outcome is warranted and ongoing at this time.

Methods

CLL Samples. The study was carried out in accordance with the institutional review board protocol approved by the Ohio State University (OSU). The initial set of CLL samples used in sequencing and real-time RT-PCR were obtained from 63 CLL patients enrolled in the CLL Research Consortium (CRC) upon written informed consent. Samples were subgrouped accordingly to their karyotype as follows: 28 samples 11q deleted, 9 samples 17p deleted, 11 samples 13q deleted, and 15 samples with a normal karyotype CLL patients. An additional 117 previously untreated CLL patients with ZAP-70 protein and methylation expression as previously described (21) were assessed for expression using similar real-time RT-PCR (these 117 samples were not sequenced but used for assessment of association between *miR-3676* expression IGVH status and ZAP-70 methylation). In total, 539 CLL samples from the CRC and OSU CLL were used for sequencing, compared with 146 normal DNAs that were extracted from leukocytes released from Red Cross leukocyte reduction filters. These 539 samples include 63 samples described above, 49 additional 17p-deleted samples, 102 CLL patient samples with familial history of cancer, and 325 additional CRC and OSU CLL samples. CLL samples were >95% composed of $CD5^+/CD19^+$ B cells. DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen). RNA was extracted using standard TRIZOL (Invitrogen) methods.

Statistical Analysis. Associations between molecular features with *miR-3676* expression were tested using two-tailed *t* tests (except one-tailed *t* test was used to assess a decrease of Tcl1 expression in samples with high *miR-3676* expression). Statistical significance was declared for tests with $P < 0.05$. Additional methods, including sequencing, cell sorting, real-time RT-PCR, DNA constructs, luciferase assays, and Western and Northern blotting, are described in *SI Methods*.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grant P01-CA81534 for the Chronic Lymphocytic Leukemia Research Consortium (to L.Z.R., J.C.B., T.J.K., and C.M.C.); Ohio State University Leukemia Specialized Program of Research Excellence Grant P50 CA140158 (to J.C.B., Y.P., and A.S.R.); The D. Warren Brown Foundation (J.C.B. and C.M.), National Institute of General Medical Sciences of the National Institutes of Health Training Grant T32GM068412 (to C.M.); The Harry T. Mangurian, Jr. Foundation (J.C.B.); The Leukemia and Lymphoma Society (J.C.M. and A.S.R.); and the European Comity, European Social Fund from Department 11, Calabria, Italy (R.P.).

1. Sgambati M, Linet M, Devesa S (2001) Chronic Lymphocytic Leukemia, Epidemiological, Familial, and Genetic Aspects. *Chronic Lymphocytic Leukemias*, Second Edition, Revised and Expanded, ed Cheson B (Marcel Dekker, New York) pp. 33–62.
2. Edelmann J, et al. (2012) High-resolution genomic profiling of chronic lymphocytic leukemia reveals new recurrent genomic alterations. *Blood* 120(24):4783–4794.
3. Döhner H, et al. (2000) Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 343(26):1910–1916.
4. Calin GA, et al. (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 99(24):15524–15529.
5. Calin GA, et al. (2008) MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc Natl Acad Sci USA* 105(13):5166–5171.
6. Pekarsky Y, et al. (2006) Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res* 66(24):11590–11593.
7. Visone R, et al. (2011) miR-181b is a biomarker of disease progression in chronic lymphocytic leukemia. *Blood* 118(11):3072–3079.
8. Pekarsky Y, Zanesi N, Croce CM (2010) Molecular basis of CLL. *Semin Cancer Biol* 20(6):370–376.
9. Herling M, et al. (2006) TCL1 shows a regulated expression pattern in chronic lymphocytic leukemia that correlates with molecular subtypes and proliferative state. *Leukemia* 20(2):280–285.
10. Teitell MA (2005) The TCL1 family of oncoproteins: Co-activators of transformation. *Nat Rev Cancer* 5(8):640–648.
11. Bichi R, et al. (2002) Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci USA* 99(10):6955–6960.
12. Efanov A, et al. (2010) CD5+CD23+ leukemic cell populations in TCL1 transgenic mice show significantly increased proliferation and Akt phosphorylation. *Leukemia* 24(5):970–975.
13. Yan XJ, et al. (2006) B cell receptors in TCL1 transgenic mice resemble those of aggressive, treatment-resistant human chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 103(31):11713–11718.
14. Enzler T, et al. (2009) Chronic lymphocytic leukemia of Emu-TCL1 transgenic mice undergoes rapid cell turnover that can be offset by extrinsic CD257 to accelerate disease progression. *Blood* 114(20):4469–4476.
15. Sánchez-Beato M, Sánchez-Aguilera A, Piris MA (2003) Cell cycle deregulation in B-cell lymphomas. *Blood* 101(4):1220–1235.
16. Scielzo C, et al. (2010) HS1 has a central role in the trafficking and homing of leukemic B cells. *Blood* 116(18):3537–3546.
17. Zanesi N, Balatti V, Bottoni A, Croce CM, Pekarsky Y (2012) Novel insights in molecular mechanisms of CLL. *Curr Pharm Des* 18(23):3363–3372.
18. Pekarsky Y, et al. (2000) Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. *Proc Natl Acad Sci USA* 97(7):3028–3033.
19. Pekarsky Y, et al. (2008) Tcl1 functions as a transcriptional regulator and is directly involved in the pathogenesis of CLL. *Proc Natl Acad Sci USA* 105(50):19643–19648.
20. Palamarchuk A, et al. (2012) Tcl1 protein functions as an inhibitor of de novo DNA methylation in B-cell chronic lymphocytic leukemia (CLL). *Proc Natl Acad Sci USA* 109(7):2555–2560.
21. Claus R, et al. (2014) Validation of ZAP-70 methylation and its relative significance in predicting outcome in chronic lymphocytic leukemia. *Blood* 124(1):42–48.
22. Ryu S, et al. (2011) Discovery of novel human breast cancer microRNAs from deep sequencing data by analysis of pri-microRNA secondary structures. *PLoS ONE* 6(2):e16403.
23. Claus R, et al. (2012) Quantitative DNA methylation analysis identifies a single CpG dinucleotide important for ZAP-70 expression and predictive of prognosis in chronic lymphocytic leukemia. *J Clin Oncol* 30(20):2483–2491.
24. Santanam U, et al. (2010) Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression. *Proc Natl Acad Sci USA* 107(27):12210–12215.
25. Cimmino A, et al. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA* 102(39):13944–13949.
26. Ozsolak F, et al. (2008) Chromatin structure analyses identify miRNA promoters. *Genes Dev* 22(22):3172–3183.
27. Borchert GM, Lanier W, Davidson BL (2006) RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* 13(12):1097–1101.
28. Canella D, Praz V, Reina JH, Cousin P, Hernandez N (2010) Defining the RNA polymerase III transcriptome: Genome-wide localization of the RNA polymerase III transcription machinery in human cells. *Genome Res* 20(6):710–721.
29. Richard P, Manley JL (2009) Transcription termination by nuclear RNA polymerases. *Genes Dev* 23(11):1247–1269.
30. Stilgenbauer S, Zenz T (2010) Understanding and managing ultra high-risk chronic lymphocytic leukemia. *Hematology (Am Soc Hematol Educ Program)* 2010:481–488.
31. Hallek M (2013) Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. *Am J Hematol* 88(9):803–816.
32. Trbusek M, Malcikova J (2013) TP53 aberrations in chronic lymphocytic leukemia. *Adv Exp Med Biol* 792:109–131.
33. Gunn SR, et al. (2008) Whole-genome scanning by array comparative genomic hybridization as a clinical tool for risk assessment in chronic lymphocytic leukemia. *J Mol Diagn* 10(5):442–451.
34. Haussecker D, et al. (2010) Human tRNA-derived small RNAs in the global regulation of RNA silencing. *RNA* 16(4):673–695.
35. Lee YS, Shibata Y, Malhotra A, Dutta A (2009) A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes Dev* 23(22):2639–2649.
36. Liu J, et al. (2014) Loss of p53 and altered miR15-a/16-1 MCL-1 pathway in CLL: insights from TCL1-Tg:p53(-/-) mouse model and primary human leukemia cells. *Leukemia* 28(1):118–128.
37. Burger JA, Chiorazzi N (2013) B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol* 34(12):592–601.
38. Ponader S, et al. (2012) The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood* 119(5):1182–1189.
39. Calin GA, et al. (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* 353(17):1793–1801.
40. Allegra D, et al. (2014) Defective DROSHA processing contributes to downregulation of MiR-15/-16 in chronic lymphocytic leukemia. *Leukemia* 28(1):98–107.