Tcl1 Expression in Chronic Lymphocytic Leukemia Is Regulated by *miR-29* and *miR-181*

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Abstract

B-cell chronic lymphocytic leukemia (B-CLL) is the most common human leukemia in the world. Deregulation of the *TCL1* oncogene is a causal event in the pathogenesis of the aggressive form of this disease as was verified by using animal models. To study the mechanism of Tcl1 regulation in CLL, we carried out microRNA expression profiling of three types of CLL: indolent CLL, aggressive CLL, and aggressive CLL showing 11q deletion. We identified distinct microRNA signatures corresponding to each group of CLL. We further determined that Tcl1 expression is regulated by *miR-29* and *miR-181*, two microRNAs differentially expressed in CLL. Expression levels of *miR-29* and *miR-181* generally inversely correlated with Tcl1 expression in the CLL samples we examined. Our results suggest that Tcl1 expression in CLL is, at least in part, regulated by *miR-29* and *miR-181* and that these microRNAs may be candidates for therapeutic agents in CLLs overexpressing Tcl1. (Cancer Res 2006; 66(24): 11590-3)

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common human leukemia in the world, accounting for ~10,000 new cases each year in the United States (1). The *TCL1* (T-cell leukemia/lymphoma 1) oncogene was discovered as a target of frequent chromosomal rearrangements at 14q31.2 in mature T-cell leukemias (2). Previously, we reported that transgenic mice expressing *TCL1* in B cells develop B-CLL (3). These results suggested that deregulation of *TCL1* may be a causal event in the pathogenesis of B-CLL. We and others also have shown that Tcl1 is a coactivator of the Akt oncoprotein, a critical molecule in the transduction of antiapoptotic signals in B and T cells (4, 5). A recent report suggested that high Tcl1 expression in human B-CLL correlates with unmutated VH status and ZAP70 positivity, suggesting that Tcl1-driven B-CLL is an aggressive form of B-CLL (6). Another study showed that the *TCL1* transgenic model replicates the immunoglobulin V region rearrangements characteristic of the aggressive, treatment-resistant form of human B-CLL (7). One of the most significant genetic factors associated with poor prognosis in human B-CLL is the chromosome 11q deletion (8). Interestingly, B-CLL samples showing 11q deletion also display higher Tcl1 expression levels (6).

MicroRNAs are a large family of highly conserved noncoding genes thought to be involved in temporal and tissue specific gene regulation (9). We recently showed that microRNA expression profiles can be used to distinguish normal B cells from malignant B-CLL cells and that microRNA signatures are associated with prognosis and progression of chronic lymphocytic leukemia (10, 11). To determine whether Tcl1 expression is regulated by microRNAs in B-CLL, we studied microRNA expression patterns and Tcl1 protein expression in 80 B-CLL samples of three types of B-CLL: indolent B-CLL, aggressive B-CLL with normal chromosome 11, and aggressive B-CLL showing 11q deletion. We choose these three types of B-CLL because a recent study suggested a differential expression of Tcl1 in these three groups (6).

Materials and Methods

CLL samples and microRNA microchip experiments. Eighty B-CLL samples were obtained with informed consent from patients diagnosed with B-CLL from CLL Research Consortium institutions. Research was done with the approval of the Institutional Review Board of The Ohio State University. Briefly, blood was obtained from CLL patients, then lymphocytes were isolated through Ficoll/Hypaque gradient centrifugation (Amersham, Piscataway, NJ) and processed for RNA extraction using the standard Trizol method. Protein extraction was carried out as previously described (12). MicroRNA microchip experiments were done as previously described (11). Each microRNA microchip contained duplicate probes, corresponding to 326 human and 249 mouse microRNA genes. Statistical analysis was carried out as previously described (13). To identify statistically significant differentially expressed microRNA, class prediction analyses were done using BRB ArrayTools developed by Dr. Richard Simm and Amy Peng Lam.

DNA constructs, transfection, Western blotting, and luciferase assay. Full-length *TCL1* cDNA including 5′ and 3′ untranslated region (UTR) cDNA was cloned into a pUSEamp vector (Upstate Biotechnology, Chicago, IL; used in Fig. 2*B*). *MiR-29b* and *miR181b* RNA duplexes were purchased from Ambion (Austin, TX). For *miR-29* luciferase assays, a fragment of the 3′ UTR of *TCL1* cDNA, including a region complimentary to *miR-29* (Tcl1), was inserted using the *Xba*I site immediately downstream from the stop codon of luciferase into pGL3 vector (Promega, Madison, WI). For *miR181* assays, full-length *TCL1* cDNA was inserted into pGL3 vector in sense (Tcl1FL) or antisense (Tcl1FLAS) orientation. Transfections were carried out as previously described (14). Firefly and renilla luciferase activities were assayed with the dual luciferase assay system (Promega) and firefly luciferase activity was normalized to renilla luciferase activity. Cell lysate preparations and Western blot analyses were carried out using anti-Tcl1 monoclonal antibody (clone 27D6) as previously described (4). Each Western filter contained reference sample. Tcl1 protein expression was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Statistically significant microRNAs differentiating CLL subtypes

MicroRNA	Chromosomal position	*P*	Fold change (aggressive chr. 11 normal/indolent)	Fold change (aggressive chr. 11 normal/aggressive chr. 11 deletion)	Fold change (aggressive chr. 11 deletion/Indolent)
miR-363	Xq26.2	<0.000001	1.69	3.15	0.54
miR-30a	6q13	0.000001	0.79	1.95	0.41
miR-19b-1	13q31.3	0.000004	0.92	1.91	0.48
miR-29c	1q32.2	0.000006	0.63	1.77	0.35
miR-146a	5q33.3	0.000044	2.02	3.26	0.62
miR-145	5q33.1	0.000054	0.81	1.21	0.67
miR-367	4q25	0.000081	1.07	2.05	0.52
miR-129-1	7q32.1	0.000082	1.15	1.40	0.82
miR-181c	19p13.12	0.000094	0.95	2.05	0.46
miR-191	3p21.31	0.000156	1.33	2.32	0.57
miR-29b-2	1q32.2	0.000175	0.64	1.47	0.44
miR-197	1p13.3	0.000255	0.96	1.38	0.69
miR-107	10q23.32	0.000298	1.13	1.97	0.58
miR-106a	Xq26.2	0.000345	1.02	1.91	0.53
miR-130b	22q11.21	0.000413	1.14	1.84	0.62
miR-320	8p21.3	0.000514	0.92	1.34	0.69
miR-215	1q41	0.000545	0.92	1.80	0.51
miR-342	14q32.2	0.000635	1.01	1.69	0.60
miR-93	7q22.1	0.000655	1.06	1.71	0.62
miR-15b	3q26.1	0.000672	1.12	2.10	0.53
miR-21	17q23.1	0.000686	1.14	2.06	0.55
miR-185	22q11.21	0.000695	1.47	1.62	0.91
miR-181a-1	1q31.3	0.000737	0.98	2.32	0.42
miR-140	16q22.1	0.000746	0.91	1.90	0.48
miR-181b-1	1q31.3	0.001647	1.04	1.99	0.52

assessed using this sample as a reference. *P* values were two tailed and calculated by Fisher's exact test.

Results and Discussion

High expression of Tcl1 correlates with aggressive B-CLL phenotype. To evaluate Tcl1 and microRNA expression in B-CLL samples, we chose three groups of B-CLL: 23 samples of indolent B-CLL, 25 samples of aggressive B-CLL, and 32 samples of aggressive B-CLL showing 11q deletion. Detailed description of the samples can be found in Supplementary Table S1. MicroRNA microchip experiments revealed that three groups of B-CLL show significant characteristic differences in microRNA expression pattern (Table 1 and Supplementary Table S2). To determine Tcl1 protein expression in three groups of B-CLL, we carried out Western blot analysis using 27D6 Tcl1 monoclonal antibody. Results of these experiments are shown in Fig. 1*A* and *B*. Tcl1 expression was assessed as low, medium, high, and very high. Our experiments revealed low levels in 15 of 23 (65%) indolent B-CLLs, in 11 of 25 (44%) aggressive B-CLLs, and in 1 of 32 (3%) aggressive B-CLLs with 11q deletions, whereas high and very high Tcl1 expression was observed in 1 of 23 (4%) indolent B-CLLs, in 14 of 25 (56%) aggressive B-CLLs, and in 24 of 32 (75%) aggressive B-CLLs with 11q deletions (Fig. 1*B*). This finding suggests that Tcl1 overexpression correlates with aggressive B-CLL phenotype ($P < 10^{-6}$) and 11q deletions ($P = 10^{-4}$). Our results are consistent with the recently published study showing that high Tcl1 expression in human B-CLL correlates with unmutated VH status and ZAP70 positivity (6).

***MiR-29* and *miR-181* target Tcl1.** To determine which microRNA(s) target *TCL1*, we used RNAhybrid software offered by Bielefeld University Bioinformatics Server and miRBase database (15). Among miR candidates targeting Tcl1, we found that *miR-29b* and *miR-181b* (Fig. 1*C*; several other sites with lower homology not shown) are also down-regulated in aggressive

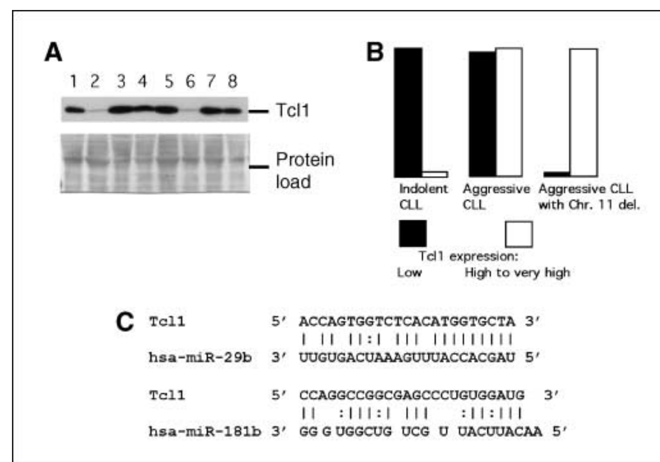

Figure 1. Tcl1 expression in B-CLL samples. *A,* Tcl1 expression in B-CLL. *Lanes 1* to *8,* B-CLL samples. *Lanes 2* and *6,* Tcl1 expression was rated as low. For all other lines, Tcl1 expression was rated as high to very high. *B,* Tcl1 expression in three groups of B-CLL. *Columns,* relative number of indicated B-CLL samples. *C,* sequence alignment of *miR-29b* and *miR-181b* and 3′ UTR of *TCL1*.

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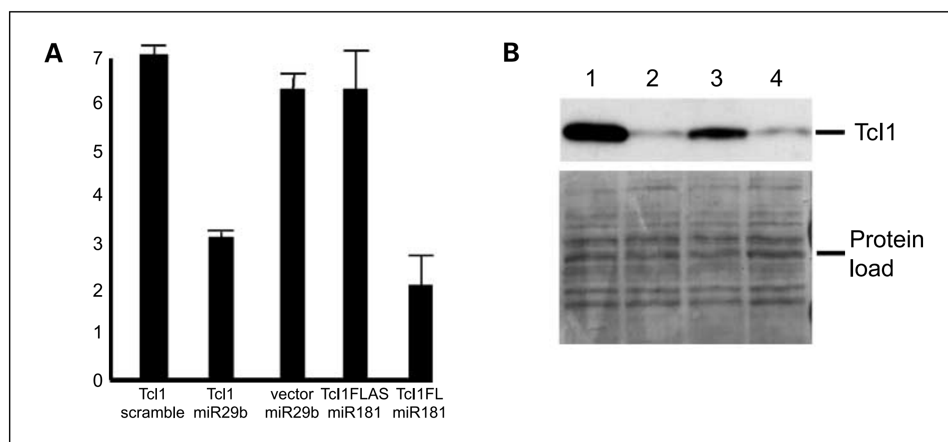

Figure 2. Tcl1 expression is regulated by *miR29* and *miR181. A, miR-29* and *miR181* target Tcl1 expression in luciferase assays. 293 cells were cotransfected with the *miR-29b* or scramble negative control, as indicated, and pGL3 construct containing a part of *TCL1* cDNA, including a region homologous to *miR-29* (Tcl1), or pGL3 vector alone as indicated. For *miR-181* assays, Tcl1FL or Tcl1FLAS were cotransfected with *miR-181*. 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. All experiments were carried out in triplicate. *B,* effect of *miR-29b* and *miR-181b* on Tcl1 protein expression. 293 cells were transfected with pcDNA3*TCL1fl* (a mammalian expression vector containing full-length *TCL1* cDNA) alone (*lane 1*) or cotransfected with pcDNA3*TCL1fl* and *miR-29b* (*lane 2*), pre-miR negative control (*lane 3*), or *miR-181b* (*lane 4*). Tcl1 expression was detected by Western blot with anti-Tcl1 antibody.

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B-CLLs with 11q deletions (Table 1). The expression of these miRs was confirmed by real-time reverse transcription-PCR in a representative set of samples (Supplementary Fig. S1). Furthermore, it was previously shown that expression of members of *miR-29* family could discriminate between CLL samples with good and bad prognosis (11). We thus proceeded to determine if these miRs indeed target Tcl1 expression using the *TCL1* 3′ UTR inserted downstream of luciferase open reading frame, as previously described (14). HEK293 cells were cotransfected with the *miR-29b* or scramble negative control, as indicated, and pGL3 construct containing a part of *TCL1* cDNA, including a region homologous to *miR-29* (Tcl1), or pGL3 vector alone as indicated. For *miR-181* assays, full-length *TCL1* cDNA was inserted into pGL3 vector in sense (Tcl1FL) or antisense (Tcl1FLAS) orientation. Figure 2*A* shows that Tcl1 mRNA expression is inhibited by *miR-29* and *miR-181*. To confirm these findings, we cloned full-length *TCL1* cDNA, including 5′ and 3′ UTRs, into cytomegalovirus mammalian expression vector and investigated whether *miR-29b* and *miR181b* affect Tcl1 protein expression levels. We cotransfected this construct with *miR-29b, miR-181b*, and pre-miR negative control (scramble) into 293 cells as indicated in Fig. 2*B*. These experiments revealed that coexpression of Tcl1 with *miR-29* and *miR-181* significantly decreased Tcl1 expression (Fig. 2*B*, *lane 2* and *4* versus *lanes 1* and *3*). We therefore concluded that *miR-29b* and *miR-181b* target Tcl1 expression at mRNA and protein levels. Interestingly, we found an inverse correlation between *miR-29b* and *miR-181b* expression and Tcl1 protein expression in B-CLL samples (Fig. 3). For samples with highest *miR-29b* expression (top 20%), 10 of 12 had low or medium Tcl1 expression, whereas in samples with highest *miR-181b* expression (top 20%), 11 of 12 had low or medium Tcl1 expression. Likewise, for samples with high expression of both *miR-29b* and *miR-181b*, 4 of 4 had low or medium Tcl1 expression. In summary, for samples with high *miR-29b* and/or *miR-181b* expression, 17 of 20 showed low or medium Tcl1 expression (P = 0.04). In addition, none of the samples with high *miR-29b* and/or *miR-181b* expression showed high Tcl1 expression (P = 0.05). These results suggest that Tcl1 expression in B-CLL is, at least in part, regulated by *miR-29* and *miR-181*.

In this report, we show that Tcl1 expression is regulated by *miR-29* and *miR-181* and this regulation is relevant to the three groups of B-CLL we studied. Although we observed a reverse correlation between Tcl1 protein expression and these two miRs, a significant proportion of B-CLL samples show low Tcl1 expression and low expression of *miR-29* and *miR-181* (Fig. 3). This suggests that, in these samples, Tcl1 expression is down-regulated transcriptionally or by other microRNAs. The fact that neither *miR-29* nor *miR-181* is located at 11q suggests that the region may contain an important regulator of the expression of these two miRs. Previously, a microRNA signature was published with 13 microRNAs that differentiate aggressive and indolent B-CLL (10, 11). Intriguingly, of the four down-regulated microRNAs in aggressive B-CLL, three are different isoforms of *miR-29* (*miR-29a-2, miR-29b-2*, and *miR-29c*), strongly suggesting that *miR-29* and *TCL1* interactions play an important role in the pathogenesis of aggressive B-CLL. Interestingly, *miR-181*

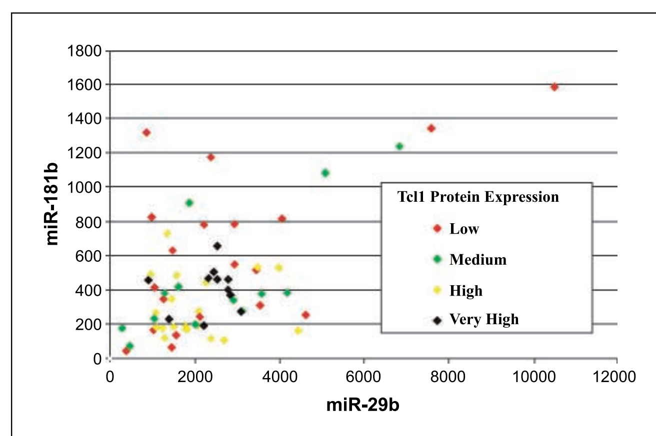

Figure 3. Inverse correlation of Tcl1 protein expression with *miR-181b* and *miR-29b* expression in B-CLL samples by microarray. The values represent microRNA microarray hybridization signal.

is differentially expressed in B cells and *TCL1* is mostly a B-cell–specific gene (16). This suggests that Tcl1 might be a target of miR-181 not only in B-CLL cells but also in normal B-lymphocytes. Additional studies are necessary to determine whether there is an inverse correlation between *TCL1* and *miR-181* expression at different stages of B-cell maturation. Because *miR-29* and *miR-181* are natural Tcl1 inhibitors, these miRs may be candidates for therapeutic agents in B-CLL-overexpressing Tcl1.

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