miR-181a/b significantly enhances drug sensitivity in chronic lymphocytic leukemia cells via targeting multiple anti-apoptosis genes

Dan-Xia Zhu1,†, Wei Zhu2,†, Cheng Fang1, Lei Fan1, Zhi-Jian Zou1, Yin-Hua Wang1, Ping Liu2, Min Hong1, Kou-Rong Miao1, Peng Liu1, Wei Xu1 and Jian-Yong Li1

1Department of Hematology and 2Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Nanjing 210029, China
†To whom correspondence should be addressed. Tel/Fax: +86 25 83781120; Email: xuweli0000@hotmail.com
Correspondence may also be addressed to Jian-Yong Li. Tel/Fax: +86 25 83781120; Email: lijianyonglm@medmail.com.cn

MicroRNAs (miRNAs) have been shown to play critical roles in regulating the progress of leukemia. We performed miRNA expression profile in six Chinese patients with chronic lymphocytic leukemia (CLL), and in peripheral B cells from pooled 30 healthy donors, using a platform containing 866 human miRNAs. The most frequent changes in miRNAs in CLL cells included downregulation of miR-126, miR-572, miR-494, miR-923, miR-638, miR-130a, miR-181a and miR-181b and up-regulation of miR-29a, miR-660, miR-20a, miR-106b, miR-142-5p, miR-101, miR-30b, miR-34a, miR-let-7e, miR-21 and miR-155. Among the miRNAs down-regulated in CLL cells, we showed that miR-181a/b expression levels were significantly lower in poor prognostic subgroups defined by unmutated immunoglobulin heavy chain variable status and p53 aberrations. Furthermore, under-expression of miR-181a and miR-181b was associated with shorter overall survival and treatment-free survival in CLL patients. We further evaluated fludarabine-induced apoptosis after transfection of primary CLL cells from 40 patients with miR-15a, miR-16-1, miR-34a, miR-181a and miR-181b mimics. Transfection of miR-34a, miR-181a and miR-181b mimics into CLL cells from p53 wild-type patients led to significant increase in apoptosis compared with miRNA control. However, enforced expression of these miRNAs had no effect on B-CLL cells from p53-mutated patients. We further demonstrated that miR-181a and miR-181b inhibiting BCL-2, MCL-1 and X-linked inhibitor of apoptosis protein by direct binding to 3’UTR. Thus, these results suggest that miR-181a/b may play important roles in the pathogenesis of CLL and may provide a possible therapeutic avenue and a sensitive indicator of the activity of the p53 axis in CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the Western world, but is less frequent in Eastern countries. Although with the aging of Chinese population and the fact that most CLL patients can live for many years, the number of people affected by CLL is growing every year. This disease has been regarded as a prime example of a tumor with low levels of cell turnover, in which an intrinsic resistance to apoptosis is responsible for the gradual accumulation of neoplastic CD5+ B cells. Consistent with this notion, CLL cells express high levels of the anti-apoptotic BCL-2 family member proteins, BCL-2 and MCL-1, which have been associated with resistance to chemotherapy, thus making this disease extremely heterogeneous (1–3). BCL-2 is a central player in the genetic program of eukaryotic cells, favoring survival by inhibiting cell death (4). MCL-1 has been strongly associated with failure to achieve a complete response to fludarabine, chlorambucil and rituximab, both in vitro and in vivo, and increasing resistance of leukemic cells to chemotheraphy-induced apoptosis is associated with induction of MCL-1 (5–7). Moreover, the X-linked inhibitor of apoptosis protein (XIAP) is also elevated in CLL cells and it contributes to apoptotic resistance (8,9). These results indicate that the apoptotic resistance of CLL cells to chemotherapeutic agents and monoclonal antibodies is, in part, related to increased levels of the anti-apoptotic proteins MCL-1, BCL-2 and XIAP. However, the exact mechanisms responsible for their up-regulation remain obscure.

MicroRNAs (miRNAs) are a recently discovered class of non-coding RNAs that control gene expression either by degradation of target miRNAs or, more commonly for animal miRNAs, by post-transcriptional repression (10,11). Many of the miRNAs map to fragile sites, minimal regions of amplification, sites of loss of heterozygosity, and common breakpoint regions in or near oncogenes and/or tumor-suppressor genes (12). Because chromosomal alterations occur in approximately 80% of CLL cases, natural deregulation of miRNAs occurs in CLL. The target genes of these miRNAs, including BCL-2, TCF-1 and MCL-1, are major components of survival pathways presumably activated in CLL cells. The first evidence of the involvement of miRNAs in CLL came from Calin et al. (13) who demonstrated that BCL-2 was regulated by the miRNAs, miR-15a and miR-16-1, located in the minimally deleted region 13q14.3 (deleted in more than 50% of CLL cases). Furthermore, MCL-1 expression has been shown to be regulated by miR-29b, and the expression of the TCF-1 oncogene has been reported to be regulated by miR-29b and miR-181b (14,15). Substantial evidence points to a role for miRNAs in the etiology and pathogenesis of CLL by targeting oncogenes or tumor suppressors.

Although CLL cells are sensitive to the chemotherapies routinely used to treat the disease, CLL remains incurable. The role of miRNAs in augmenting the apoptotic effects of chemotherapy is thus important. In this study, we investigated the miRNA expression profile in malignant B cells isolated from Chinese patients with CLL. MiR-181a and miR-181b levels were monitored in a cohort of 156 well-characterized CLL patients. We also examined the effects of modulating miRNA levels in primary CLL cells. The results indicated that miRNA deregulation is a common event in CLL, and that miR-181s could act as endogenous regulators of multiple oncogene expression, thereby inducing apoptosis.

Materials and methods

Patient selection and CLL sample processing
A total of 156 previously untreated CLL patients or patients who had not been treated during the previous 6 months were enrolled in this study between December 2008 and May 2011. In this study, 142/156 patients had received no chemotherapy at all. The diagnosis and response were based on the revised National Cancer Institution criteria (16). All patients had a lymphocytosis of at least $5 \times 10^9/l$ with $>$85% B-lymphocytes with monoclonal surface immunoglobulin (dim), CD20 (dim), CD5, CD19 and CD23 expressions. Mutational status of the immunoglobulin heavy chain variable (IGHV) region gene and the status of p53 mutations (exons 2–11) were determined as we described previously (17,18). Genomic aberrations were detected by interphase FISH analysis using a commercial probe set (CLL panel; Vyssis), as described previously (19). The cutoff level for positive values (mean of normal control ±3 SD), determined from samples of eight cytogenetically normal persons, was 7.5% for ATM deletions and 10% for TP53 deletions, respectively.

For preparation of primary cell cultures, CLL cells from 40 untreated patients were isolated from heparinized venous blood by density gradient centrifugation. The isolated cells were predominantly B cells (>85% CD5+/CD19+), as assessed by flow cytometry (FACScan, Becton Dickinson). Freshly isolated CLL B cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO2 at 37°C.
miRNA microarray assay

Total RNA was extracted from primary CLL B cells and purified CD19+ (>95%) normal B lymphocytes using Trizol (Invitrogen). About 5 µg of total RNA was used for hybridization of miRNA microarray chips containing 866 human miRNAs (Agilent). Oligonucleotide arrays were printed with trimer oligonucleotide probes (antisense to miRNAs) specific for the 866 miRNAs on GeneScreen Plus membranes (NEN Life Science Products). Hybridization signals were detected by biotin-containing transcripts. Each labeled RNA sample was hybridized with three separate membranes to ensure the accuracy of the hybridizations. Processed slides were scanned using a microarray scanner (Axon Instruments). Raw data were normalized and analyzed using GeneSpring software (Agilent). A greater than 2.0-fold increase in expression level was set as the threshold indicating significant change. Multiple testing using the Bonferroni correction was used as a confidence filter for differential miRNAs.

Real-time quantitative reverse transcription–PCR for miR-181a and miR-181b

Total RNA was isolated using Trizol reagent and the miRNAs were reversely transcribed into cDNAs. The reaction mixture contained 1 µg of purified total RNA, 5× M-MLV buffer (Invitrogen), M-MLV (200 U/µl, Invitrogen) 1.0 µl, dithiothreitol 1.0 µl, stem-loop reverse transcription (RT) primer (Supplementary Table 1 is available at Carcinogenesis Online) (10 pmol/µl) 1.0 µl, RNase inhibitor (40 U/µl) 0.5 µl and dNTP (10 mmol/l) 1.0 µl. In this study, 20 µl reactions were performed with the following thermal cycling parameters: 60 min at 42°C, 5 min at 80°C and then held at 4°C. Reactions for quantitative reverse transcription–PCR (qRT–PCR) were conducted in triplicate, using the Applied Biosystems ABI 7300 Real-time PCR system. Each reaction mixture contained 10 µl of RT product (normalized to 500 ng), 12.5 µl of SYBR GREEN PCR Master Mix, 0.5 µM of each primer and denatured water to a total volume of 25 µl. Reactions were run with the following thermal cycling parameters: 95°C for 5 min followed by 35 cycles of 95°C for 5 s and 60°C for 30 s, melting curve program (60–95°C) with a heating rate of 0.1°C/s. Relative expression was calculated using the comparative Ct method. Each sample was normalized based on its endogenous U6 RNA content. Sequences of amplified production were verified by DNA sequencing.

Exogenous overexpression of mature miRNAs through transient transfection

Freshly isolated primary CLL cells were plated in 24 well plates (5 × 10⁵ cells per well) and transiently transfected with 100 nM of mature miRNA mimics of miR-15, miR-181a, miR-181b, and mature miR-181a and 100 nM of a negative control random miRNA (GenePharma Company, Shanghai, China) using siPORT neoFX Transfection Agent (Ambion, Austin, TX), according to the manufacturer’s protocol.

Dual luciferase activity assay

The 3’UTRs of human BCL-2, MCL-1 and XIAP cDNA containing the putative target sites for the mature miR-181s (miR-181a-d) were chemically synthesized and inserted at the XbaI site, immediately downstream of the luciferase gene in the pGL3-control vector (Promega, Madison, WI) by Biomics Biotechnologies Co., Ltd (Nantong, China). Twenty-four hours before transfection, cells were plated at 5 × 10⁵ cells per well in 24 well plates. About 200 ng of pGL3-BCL–UTR, pGL3-MCL–UTR and pGL3-XIAP–3’UTR were each transfected in combination with 80 ng pRL-TK (Promega). The miRNAs used for cotransfection were 100 nM of miR-181a, miR-181b, anti-miR-181a, anti-miR-181b and miRNA mimic-negative control. Light emissions by firefly and Renilla luciferase activities were measured consecutively using dual luciferase assays (Promega) 24 h after transfection. The ratios of Renilla versus firefly signals served as a measure of reporter activity normalized for transfection efficiency. Three independent experiments were performed in triplicate.

Western blotting

Cells were harvested and homogenized with lysis buffer 72 h after transfection with miRNA mimics (including miR-181a, miR-181b and negative control miRNA mimics). Total protein was separated by denaturing 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western analysis was performed as described. Primary antibodies for BCL-2, GAPDH and MCL-1, XIAP, and TCL-1 were purchased from Bioworld Technology and Cell Signaling Technology, respectively. Protein levels were normalized to GAPDH. Fold changes were determined.

Fig. 1. Hierarchical clustering of miRNA in CLL samples. CLL samples were clustered according to the expression profile of 31 twofold differentially expressed miRNAs between six CLL patients and three pooled controls. The level of miRNA expression is color-coded. Red, higher miRNA expression; green, lower miRNA expression; black, no difference. N: controls; P: CLL patients.
Apoptosis assay
Twenty-four hours after transfection as described above, CLL cells were treated with fludarabine at a final concentration of 3.5 µmol/l. After a further 48 h, cells were labeled with CD19-allophycocyanin (BD Biosciences) and then resuspended in 500 µl binding buffer containing 5 µl annexin V-fluorescein isothiocyanate and 10 µl propidium iodide (Bestbio, Shanghai, China). Apoptosis of CD19+ CLL cells was quantified using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Statistical analysis
The relative amounts of miRNA-181a/b were calculated using the equation $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct$ was calculated by subtracting the Ct for U6 small nuclear RNA from those for miRNA-181a/b. Correlations between miRNA expression and TP53 status were assessed using Mann–Whitney non-parametric tests. Receiver operating characteristic (ROC) curves were analyzed using GraphPad Prism 5.0 software (GraphPad Software) to determine the miR-181a and miR-181b expression cutoff values that best distinguished between mutated and unmutated IGHV cases. Overall survival (OS) and treatment-free survival (TFS) were estimated using the Kaplan–Meier method and were compared by log-rank tests. Paired t-tests were used to compare the apoptotic rates between control and miRNA-transfected samples from the same patient. All tests were two-sided. An effect was considered to be statistically significant at $P < 0.05$. Statistical analyses were performed with GraphPad Prism Version 5.0 (GraphPad Software).

Results
MiRNA expression profile in Chinese patients with CLL
The miRNA expression signature in Chinese patients with CLL was identified using purified B cells from CLL patients ($n = 6$) and healthy controls ($n = 3$). All the CLL patients were treatment-naive, and pooled samples from every 10 normal healthy donors were used as a common reference for CLL cases. The majority of patients presented with Binet stage A and showed a favorable risk profile, as assessed by clinical and molecular features (Supplementary Table 2 is available at Carcinogenesis Online). RNAs isolated from CLL cells and normal lymphocytes were hybridized to a customized miRNA microarray platform containing 866 human miRNAs. Unsupervised hierarchical clustering revealed clearly different expression patterns between CLL and normal lymphocytes (Figure 1). A total of 18 miRNAs were upregulated and 13 were significantly down-regulated in CLL cells, on the basis of the basis of a threshold of a median fold change greater than 2.0, a minimal absolute difference of signal intensity defined by the detection threshold level, and a $P$-value < 0.05 (Figure 1).

MiRNA-181a/b expression are associated with adverse outcomes in patients with CLL
qRT–PCR was performed in 156 CLL patients to verify the miRNA microarray data and determine the prognostic value of miR-181a and miR-181b. qRT–PCR verified that miR-181a/b were downregulated in CLL cells compared with normal lymphocytes (Supplementary Figure 1 is available at Carcinogenesis Online). Furthermore, they were more significantly reduced in patients with unmutated IGHV genes (Supplementary Figure 2 is available at Carcinogenesis Online). Using mean relative expression level as a cutoff value, we analyzed the association between OS and TFS in CLL patients with miR-181a/b expression. The cutoff determination was based on ROC analysis, optimizing concordance with IGHV mutational status with samples

Fig. 2. miR-181a and miR-181b can predict OS and TFS. The representative OS and TFS curves for miR-181a (A and C) and for miR-181b (B and D). miR-181a and miR-181b were measured by qPCR, and cutoffs were optimized to maximize IGHV mutational concordance using ROC curve analyses. Statistical differences between curves were calculated using the log-rank test.
with a $2^{-\Delta\text{Ct}}$ value <6.70 and <3.35 representing low expression of miR-181a and miR-181b, respectively (Supplementary Figure 3 is available at Carcinogenesis Online). A total of 10 patients (6.25%) in the current series died (CLL-related deaths) within a median follow-up period of 29 months (range, 2–160 months). Thus, as shown in Figure 2A and B, down-expressions of miR-181a and miR-181b levels were significantly associated with reduced OS ($P = 0.0235$ and $P = 0.012$, respectively). A total of 142 patients were untreated at enrollment, but 28 patients received fludarabine-based treatment during follow-up because of disease progression according to International Workshop on Chronic Lymphocytic Leukemia criteria (16).

Monoallelic TP53 inactivation was associated with poor prognosis, and survival was poor in patients with del(17p13), del(11q22.3) or TP53 mutation. We defined a ‘p53-attenuated’ group, including patients with del(17p13), del(11q22.3) or TP53 mutations, and investigated correlations between miR-181a and miR-181b expression and p53 status. A combination of results from FISH and TP53 mutation analyses in cases for which both types of data were available ($n = 133$) demonstrated high expression of miR-181a and miR-181b in samples from the wild-type p53 cohort, whereas CLL cells from ‘p53-attenuated’ ($n = 41$), ‘del(17p13) only’ ($n = 6$), or ‘p53 mutations only’ ($n = 13$) patients consistently showed low expression of miR-181a/b (Figure 3).

**MiR-181a/b modulate expression of anti-apoptotic proteins in CLL B cells**

Several anti-apoptotic proteins, including BCL-2, MCL-1, TCL-1 and XIAP, are elevated in CLL cells and contribute to apoptotic resistance. To ascertain if miR-181a/b regulate these predicted targets, we transfected primary CLL cells from 20 patients (with reduced endogenous miR-181a/b levels) with mature miRNA mimics of miR-181a and miR-181b, respectively. Control experiments were performed in the same manner. Increased expression of miR-181a/b following transfection was verified by real-time RT–PCR. Significant changes in BCL-2, MCL-1, TCL-1 and XIAP levels were found 72 h after transfection with mature miR-181 mimics, compared with transfection with control miRNA mimic, respectively (Figure 4).

---

**Fig. 3.** Correlations between miR-181a/b expression and TP53 status. (A and B) miR-181a and miR-181b expressions were extremely downregulated in the patients with ‘p53-attenuated’ compared with patients with ‘p53 wild-type’. (C and D) CLL cells from ‘p53-attenuated’ [including patients with del(17p13) and/or del(11q22) and/or with mutation of TP53], ‘del(17p13) only’ and ‘p53 mutations only’ patients consistently showed low expression of miR-181a and miR-181b.
MiR-181 acts directly at the BCL-2/MCL-1/XIAP-3’UTR

TargetScanHuman 5.1 (http://www.targetscan.org) was used for the prediction of the miR-181s targeted genes. The sequence alignment of the miR-181s with MCL-1, XIAP and BCL-2–3’UTR of different species is conserved, which indicates that MCL-1, XIAP and BCL-2 are the common target genes of the miR-181s. We found that in SGC7901/VCR and A549/CDDP cells, a significant decrease in relative luciferase activity was noted when pGL3-MCL-1-3’-UTR, pGL3-XIAP-3’-UTR or pGL3-BCL-2–3’-UTR was cotransfected with the miR-181s mimics but not with the miRNA mimic control, respectively, whereas transfection with anti-miR-181a or anti-miR-181b inhibitor increased the luciferase reporter activity of pGL3-BCL-2, pGL3-MCL-1 and pGL3-XIAP constructs in cells (Figure 5A and B). Thus, miR-181a and miR-181b directly inhibit the expression of BCL-2, MCL-1 and XIAP by binding to the target sequence.

MiR-181a/b significantly sensitizes CLL cells to fludarabine-induced apoptosis

The development of drug resistance in various cancer cells has been linked to a reduced susceptibility to drug-induced apoptosis, which was shown to result, at least in some cases, from overexpression of anti-apoptotic proteins. We suggest that the miR-181 family might play a role in the development of multidrug resistance by modulating apoptosis via targeting BCL-2, MCL-1 and XIAP. To confirm this hypothesis, we evaluated fludarabine-induced apoptosis after transfection of primary CLL cells from 40 patients with mature miR-15a, miR-16-1, miR-34a, miR-181a or miR-181b mimics, and a control miRNA mimic. A marked increase in apoptosis, as assessed by flow cytometry, was observed in miR-181a- and miR-181b-mimic transfected primary CLL cells after fludarabine treatment, compared with control miRNA mimic-transfected cells (Supplementary Figure 4A is available at Carcinogenesis Online). As reported by several groups (13,23,30,31), enforced expression of miR-15a, miR-16-1 and miR-34a also increased apoptosis, though the effects seemed weaker than that of the miR-181s. Furthermore, the tumor-suppressive effect of miR-181b in CLL cells was more apparent than that of miR-181a.

Primary CLL samples from 40 patients were then grouped according to their p53 attenuation status, as described above. Transfection of miR-181a and miR-181b mimics in 30 patients without p53 attenuation led to significant apoptosis increase within 48h, in comparison to transfection with the control. Interestingly, overexpression of miR-181a and miR-181b had no significant effect in CLL cells from 10 p53-attenuated patients [defined as patients with del(17p13), del(11q22.3) or TP53 mutations]; the detailed characterization of the p53 status was shown in Table I (Supplementary Figure 4B and C is available at Carcinogenesis Online). These findings indicate that the apoptosis-inducing effects of miR-181a/b may also require p53 activation.

Discussion

This study provides the first description of a novel set of 31 miRNAs significantly deregulated in Chinese patients with CLL. Two members of the miR-181 family, miR-181a and miR-181b, are known to be the key regulators of B-cell maturation and were of specific interest. We performed qRT–PCR and confirmed the down-regulation of miR-181a and miR-181b in CLL. The results also showed that miR-181a and miR-181b expressions were dramatically decreased in patients with IGHV unmutations. Similar results were obtained comparing ‘TP53 normal’ and ‘TP53-attenuated’ patients, indicating that lower miR-181a and miR-181b expression levels were significantly correlated with poor prognosis. miR-181a and miR-181b were also shown to predict OS and TFS. The cutoff values were based on ROC analysis optimizing concordance with IGHV mutational status, representing one of the most robust molecular prognostic factors currently used.
Although this cutoff point needs to be validated in an extended patient cohort, the current results suggest that lower expression levels of these two miRNAs are linked to disease aggressiveness.

In light of the fact that p53 status is the strongest indicator of prognosis in CLL, the relationships between miR-181a/b expression and the presence or absence of abnormal p53 were explored, and lower miR-181a/b expression was found in ‘p53-attenuated’ patients compared with patients without p53 attenuation. In our cohorts, CLL cases with the TP53 mutation had significantly lower miR-181a/b levels, even in the absence of deletions in the TP53 gene, demonstrating that TP53 mutational status may also impact on miR-181a and miR-181b expression. The p53-activating kinase, ATM, which is absent from one allele in most patients with del(11q22.3), also influences p53 function. This study also detected a tendency toward lower miR-181a and miR-181b levels in patients with del(11q22.3).

It is generally accepted that the intrinsic cell death pathway or the mitochondrial pathway plays a predominant role in chemotherapy-induced apoptosis. In addition, a number of pro-survival signaling pathways are known to be constitutively activated in CLL cells, including the STAT3, PI3K and NF-κB pathways (20–22). This study aimed to identify some of the major mRNA targets and signaling pathways that mediate miR-181a/b regulation in CLL cells. BCL-2, MCL-1, TCL-1, XIAP and k-RAS, which are elevated in CLL cells and contribute to apoptotic resistance, were identified as potential targets of miR-181a and miR-181b. BCL-2 is known to be a critical regulator of the mitochondrial pathway by diminishing cytochrome release, leading

**Table I.** Results of karyotyping, FISH detection and TP53 sequencing for p53 status in 10 CLL patients with ‘p53 abnormal’

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age</th>
<th>Binet stage</th>
<th>VH</th>
<th>VH-gene</th>
<th>CLL genetics</th>
<th>Other hierarchical cytogenetics</th>
<th>TP53 mutation details</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>del(17p13)</td>
<td>del(11q22)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M/69</td>
<td>C</td>
<td>Mutated</td>
<td>IGHV3-23*05</td>
<td>NA</td>
<td>Yes (14%) No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>F/48</td>
<td>C</td>
<td>Unmutated</td>
<td>IGHV7-4-1*02</td>
<td>Complex</td>
<td>Yes (25%) No</td>
<td>del6q23</td>
</tr>
<tr>
<td>3</td>
<td>F/66</td>
<td>C</td>
<td>Unmutated</td>
<td>IGHV4-4*02</td>
<td>Complex</td>
<td>Yes (37%) No</td>
<td>del13q, t(14q32)</td>
</tr>
<tr>
<td>4</td>
<td>F/53</td>
<td>C</td>
<td>Mutated</td>
<td>IGHV4-4*07</td>
<td>11p+</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M/57</td>
<td>A</td>
<td>Mutated</td>
<td>IGHV4-59*01</td>
<td>Normal</td>
<td>Yes (22%) No</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M/73</td>
<td>B</td>
<td>Unmutated</td>
<td>IGHV1-3*01</td>
<td>Complex</td>
<td>Yes (15%) Yes (23%) No</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M/56</td>
<td>C</td>
<td>Unmutated</td>
<td>IGHV3-48*01</td>
<td>Complex</td>
<td>Yes (18%) No</td>
<td>+12</td>
</tr>
<tr>
<td>8</td>
<td>M/61</td>
<td>C</td>
<td>Unmutated</td>
<td>IGHV7-4-1*02</td>
<td>Normal</td>
<td>Yes (13%) No</td>
<td>del6q23, del13q, t(14q32)</td>
</tr>
<tr>
<td>9</td>
<td>M/52</td>
<td>B</td>
<td>Unmutated</td>
<td>IGHV3-74*01</td>
<td>Normal</td>
<td>Yes (17%) Yes (92%) del13q</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>F/67</td>
<td>C</td>
<td>Mutated</td>
<td>IGHV4-34*01</td>
<td>Complex</td>
<td>Yes (91%) No</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 5.** Dual luciferase assay performed in SGC7901/VCR and A549/CDDP cells suggested that BCL-2, MCL-1 as well as XIAP are the target genes of both miR-181a and miR-181b. The cells were seeded and then co-transfected with pGL3-BCL-2, pGL3-MCL-1 or pGL3-XIAP reporter construct and miR-181a/b mimics or anti-miR-181a/b inhibitor for 24h. Relative luciferase activity was compared with those transfected with miRNA control mimics. Data are expressed as mean ± SD of three independent experiments.
to inhibition of apoptosis. Previous studies revealed that miR-15a and miR-16-1 directly regulate BCL-2 expression, thereby modulating the susceptibility of cancer cells to chemotherapeutic drug-induced apoptosis (13,23). MCL-1, a downstream target of activated STAT3, appears to be dynamically regulated by a wide array of cellular miRNAs in most CLL cells, including ligation of the B-cell receptor and signaling via CD40, vascular endothelial growth factor and the PI3K/AKT pathway (24–26). XIAP is an anti-apoptotic protein that is elevated in CLL, and is also a downstream target of the NF-κB pathway, which modulates chemosensitivity through its ability to inhibit apoptosis downstream of the caspase-8 and caspase-9 cascades (27). The results of this study clearly indicate that the downregulation of miR-181a and miR-181b in CLL cells is functionally significant, because modulation of their expression altered the sensitivity of cells to chemotherapeutic drugs and confirmed previous suggestions that the expression levels of a few miRNAs could sensitize cancer cells to drug-inducing cell death.

Although exogenous overexpression of miR-34a, miR-181a and miR-181b significantly increased apoptosis in most patients, no response to exogenous overexpression of miR-34a, miR-181a and miR-181b was detected in some CLL patient samples. All non-responders belonged to the p53-attenuated group, suggesting that miR-181a/b may require an intact p53 pathway to exert their continuous apoptosis-inducing effects. Recently, bioinformatics has identified a few conserved regions within the genes encoding miR-181 family members that contain several matches to the canonical p53 binding sites (28). Further, Cervo et al. reported the SIRT1 as the direct target of miR-138, miR-181a and miR-181b (29). The results of the current and previous studies suggest that miR-181a/b may function in a similar way to miR-34a (30,31): transcription of pri-miRNA-181a and -181b is directly activated by p53, and these miRNAs then promote cell cycle arrest and apoptosis by targeting multiple anti-apoptosis genes. On the other hand, despite its role as a p53 downstream target, miR-34a, miR-181s also seems to influence p53 upstream factors, leading to p53 activation via a positive feedback loop. For example, it has been demonstrated that miR-34a and miR-181a/b suppress the p53 suppressor SIRT1, resulting in increased p53 acetylation and activation (32). Then, we hypothesized that if the p53 is attenuated, the positive feedback pathway may be blocked, and the apoptosis-inducing effects of miR-181s may be eliminated. However, further studies are needed to confirm the existence of this pathway. This study demonstrated that miR-181a and miR-181b are potent regulators in primary CLL cells and inhibit specific pro-survival pathways known to be relevant to CLL cell biology. Function research of miR-181s was first focused on hematopoietic lineage differentiation in mouse, mmu-miR-181s was reported to show an obviously high expression at the adult stage, compared with embryonic and early postnatal stages (33). Studies by Fanini et al. (34) showed that miR-181a and miR-181b may serve as tumor suppressors in human acute myeloid leukemia and agents that increase miR-181a expression-induced apoptosis of acute myeloid leukemia blasts. Besides this, miR-181b could negatively regulate activation-induced cytidine deaminase in B cells and plays a role in preventing B-cell malignant transformation (35). Moreover, recent study by Rossi et al. (36) also showed that low-expression level of miR-181b suggested therapy-refractory in patients with CLL. We therefore speculated that miR-181a/b might function to regulate the differentiation state of human lymphocytes. Downregulated miR-181a/b might be correlated with the de-differentiated state of human lymphocytes, or even with their aggressive nature, thus contributing to disease progression and multidrug resistance.

In conclusion, this study identified the predominant miRNA expression signature of Chinese patients with CLL. We also determined that miR-181a/b expressions levels were significantly correlated with unfavorable prognostic factors and were predictors of OS and TFS. The fludarabine-sensitizing effects of miR-181a/b were associated with down-regulation of the anti-apoptotic proteins BCL-2, MCL-1 and XIAP, which are characteristically associated with leukemic cell resistance to chemotherapeutic agents. Our results further demonstrated that miR-181a/b expression was correlated with TP53 status, and that the apoptosis-inducing effects of miR-181a/b may require p53 activation. These data suggest an important role for miR-181a/b in the pathophysiology of CLL. Although further studies are needed to confirm these results, it is possible that miR-181a/b may provide surrogate markers for p53 functionality. Further potential therapeutic applications of multidrug resistance-related miRNAs (such as miR-15a/16-1, miR-34a and miR-181a/b) should be investigated in future studies, together with further consideration of the miRNA/p53 feedback loop.

**Supplementary material**

Supplementary Tables 1 and 2 and Figures 1–4 can be found at http://carcin.oxfordjournals.org.

**Funding**

National Natural Science Foundation of China (30871104, 30971296, 81000216, 811070488, 811070485, 811070486 and 81100352); Natural Science Foundation of Jiangsu Province (BK2010584); ‘Qing Lan’ project of Jiangsu Province; ‘Liu Da Ren Cai Gao Feng’ of Jiangsu Province; University Doctoral Foundation of the Ministry of Education of China (20093234120007 and 20093234110010); the key Projects of Health Department of Jiangsu Province (KZ201108); the Program for Development of Innovative Research Team in the First Affiliated Hospital of NJMU; the Project of Innovation in Postgraduate of Jiangsu Province; the Program for Innovative Team of Science and Technology and Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions; Jiangsu Province Higher Education Institute Foundation of Science and Technology Innovation Team Programme.

**Acknowledgements**

Conflict of Interest Statement: The authors declare no conflict of interest.

**References**


Received May 15, 2012; revised April 14, 2012; accepted May 11, 2012