MicroRNA dysregulation to identify therapeutic target combinations for chronic lymphocytic leukemia

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Loss of *miR-15/16* is the most common genetic lesion in chronic lymphocytic leukemia (CLL), promoting overexpression of *BCL2*, which factors in leukemia pathogenesis. Indeed, an inhibitor of Bcl2, venetoclcax, is highly active in the treatment of patients with CLL. However, single-agent venetoclax fails to eradicate minimal residual disease in most patients. Accordingly, we were interested in other genes that may be regulated by *miR-15/16*, which may target other drivers in CLL. We found that *miR-15/16* targets *ROR1*, which encodes an onco-embryonic surface protein expressed on the CLL cells of over 90% of patients, but not on virtually all normal postpartum tissues. CLL with high-level expression of ROR1 also have high-level expression of Bcl2, but low-to-negligible *miR-15/16*. Moreover, CLL cases with high-level ROR1 have deletion(s) at the chromosomal location of the genes encoding *miR-15/16* (13q14) more frequently than cases with low-to-negligible ROR1, implying that deletion of *miR-15/16* may promote overexpression of *ROR1*, in addition to *BCL2*. ROR1 is a receptor for Wnt5a, which can promote leukemia-cell proliferation and survival, and can be targeted by cirmtuzumab, a humanized anti-ROR1 mAb. We find that this mAb can enhance the in vitro cytotoxic activity of venetoclax for CLL cells with high-level expression of ROR1, indicating that combining these agents, which target ROR1 and Bcl2, may have additive, if not synergistic, activity in patients with this disease.

CLL | ROR1 | miR-15/16 | Blc2 | venetoclax

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries (1). Although there is no single genetic lesion responsible for leukemogenesis, the CLL cells of most patients have genetic aberrations detectable by fluorescence in situ hybridization (FISH) (2), the most frequent being deletion of 13q14, which is observed in over half of all cases (3). Studies on this and familial CLL revealed that the critical gene(s) deleted/dysregulated on chromosome 13 actually encoded two microRNA, *miR-15a* and *miR-16-1*, providing evidence that microRNA can contribute to human disease (4, 5). Moreover, these studies found *miR-15/16* down-regulated in the CLL cells of most patients (4, 5). Subsequent studies found that *miR-15/16* targeted *BCL2* (6), a gene discovered in 1984 in studies on follicular lymphoma (7). Down-regulation of *miR-15/16* allowed for the high-level expression of Blc2, which is observed in the CLL cells of most patients and accounts for resistance of CLL cells to spontaneous or drug-induced apoptosis (6). Thus, Fesik and coworkers (8) at Abbott, developed a protein/protein interaction inhibitor of Blc2. This inhibitor, however, also targeted other members of the Blc2 family, including BclXL, which is essential for platelet survival (9, 10). More recently, Abbott modified the inhibitor to target only Blc2 (11). Because this drug, named ABT-199 (venetoclax) can induce complete responses in patients who were refractory to chemotherapy (12, 13), the FDA approved venetoclax for treatment of patients with relapsed/refractory CLL (14).

T.J.K.'s laboratory and others showed that the receptor tyrosine kinase-like orphan receptor 1 (ROR1) is an onco-embryonic antigen expressed on the surface of CLL cells, but not on cells of healthy adults, except a small subgroup of distinctive pro-B cells,

named hematogones (15–20). Evaluation of the CLL cells from 1,568 cases showed that levels of ROR1 varied on the leukemia cells of different patients (21). Although the vast majority of cases expressed detectable ROR1, 5–10% of cases expressed negligible levels of ROR1, comparable to that of normal B cells. Moreover, patients with CLL cells that had high-level ROR1 had more aggressive disease and shorter overall survival than patients with CLL cells having low-level ROR1 (21). ROR1 is a receptor for Wnt5a (20), which can enhance chemokine-directed migration and growth/survival of CLL cells (22). Moreover, antibodies targeting ROR1 can inhibit ROR1-dependent Wnt5a-induced cell growth/survival of leukemia cells expressing ROR1 and Tcl1, which were found associated with aggressive disease (23). Thus, T.J.K.'s laboratory developed an anti-ROR1 antibody (cirmtuzumab) for clinical trials (24). In this study, we examined for microRNAs that potentially could contribute to the noted differences in the expression of ROR1 observed in the CLL cells of patients with this disease.

Results

MicroRNA Signatures. We compared the microRNA profile of CLL cases with low expression of ROR1 with that of cases with high expression of ROR1. We performed Nanostring analysis on 24 CLL cell samples (12 ROR1 low and 12 with ROR1 high), as described (21) to assess the microRNA expressed by CLL B cells with high- versus low-level expression of ROR1. All 17 samples from cohort A were used for this experiment. Additional samples

Significance

This study highlights a pathogenic role for loss of *miR-15/16* in chronic lymphocytic leukemia (CLL). We find that loss of mi*R-15/16* promotes overexpression of *BCL2* and *ROR1*, which each factor in leukemia pathogenesis. Treatment with single-agent venetoclcax, which inhibits Bcl2, is highly effective in patients with CLL, but generally cannot eradicate minimal residual disease. Since loss of *miR-15/16* also promotes expression of *ROR1*, we examined the cytotoxic activity of venetoclax in combination with an anti-ROR1 mAb, cirmtuzumab. We found that cirmtuzumab could enhance the in vitro cytotoxicity of venetoclax for CLL cells with high-level ROR1. This suggests that combining agents that target ROR1 and Bcl2 may be highly effective in the treatment patients with this disease.

were chosen randomly from the other two cohorts: Two were selected from cohort B and five from cohort C (Table S1). We found that CLL samples with high- versus low-ROR1 expression clustered into two subgroups based on differential expression of several microRNAs. Table 1 shows the signature of 17 microRNAs that significantly differ in their expression levels between ROR1-high versus ROR1-low samples. Among the most down-regulated microRNAs in ROR1-high CLL relative to ROR1-low CLL were *miR-15a* and *miR-16-1*, suggesting that loss of these two microRNAs contributes to the overexpression of ROR1. The values for the expression levels of *miR-15a* and *miR-16-1* were highest for cases with low-to-negligible expression of ROR1, providing further evidence of the role of these microRNAs in the regulation of *ROR1*. Other microRNAs found low-to-absent in CLL cells that expressed high levels of ROR1 were *miR-365a/b* (also found to be targeting *ROR1* as shown in Table 1), *miR-451*, and *miR-199a/b* (not predicted to target *BCL2* or *ROR1*). We confirmed these data by performing a real-time PCR for *miR-15a* and *miR-365* on the samples used for nanostring. Interestingly, the combined *miR-15* and *miR-16* expression was the highest among all differentially expressed microRNAs and almost 20-fold higher than *miR-365* (Table 1). Both *miR-15a* and *mir-365* showed lower expression levels in ROR1-high samples compared with ROR1-low samples (Fig. S1).

MiR-15/16 and miR-365 Target ROR1. Since target prediction by TargetScanHuman 7.0 software predicted that *miR-15/16* or *miR-365a-3p* could target *ROR1* (Fig. S2), we focused our study on these microRNAs. We performed a luciferase assay by cotransfecting HEK-293 cells with a vector expressing either the WT or the mutated version of the 3′UTR of *ROR1* and either *premiR-15a-5p*, *premiR-16-5p*, *premiR-365a-3p*, or the scramble negative control 1 (NC1). As shown in Fig. 1, both targets were confirmed. Cotransfection of pSI-*ROR1*-WT and either *premiR-15a-5p*, *premiR-16-5p*, or *premiR-365a-3p* caused over a twofold loss in activity, while mutated versions of the construct did not (Fig. 1). To validate these findings, we performed a luciferase assay by cotransfecting HEK-293 cells with the vector expressing the WT 3′UTR of *ROR1* and two unrelated microRNAs, *premiR-210* or

premiR-4301, and observed no changes in target-gene expression (Fig. S3).

Bcl2 Expression in ROR1-High and ROR1-Low CLL. Since the loss of *miR-15/16* correlates with overexpression of Bcl2 (6), we proceeded to study the expression pattern of Bcl2 in ROR1-high versus ROR1-low CLL. We first determined ROR1 expression levels by flow cytometry in CLL B cells collected from the 17 CLL cases from cohort A, as described (21). We observed that the expression level of ROR1 inversely correlated with the expression levels of *miR-15a* and *miR-16-1-5p* (Fig. 2 A and B). CLL cases with leukemia cells expressing low levels of ROR1 had significantly higher expression levels of *miR-15/16* than CLL cases with leukemia cells expressing high levels of ROR1 (Fig. 2C). Next, since the loss of *miR-15/16* correlates with overexpression of Bcl2 (6), we examined Bcl2 expression levels in control cells and CLL cells with high versus low expression of ROR1 (Fig. S4). CLL cells expressing high levels of ROR1 also expressed high levels of Bcl2, whereas CLL cells with low ROR1 expressed low Bcl2. Fig. 2D shows the correlation of the expression levels of Bcl2 versus ROR1 obtained by assaying 12 samples from cohort A by immunoblot. The densitometry analysis is plotted in Fig. 2E, which shows the protein expression of ROR1 or Bcl2 relative to β-actin for both ROR1-high and ROR1-low samples. Fig. 2F shows the correlation between the expression levels of Bcl2 and ROR1, as assessed by flow cytometry. Similarly, Fig. 2G shows the correlation between ROR1 and Bcl2 expression analyzed by flow cytometry in the samples from cohort A. This experiment shows that CLL cells with high-level expression of ROR1 expressed significantly higher levels of Bcl2 than CLL cells with low-level expression of ROR1 (Fig. 2H). These data were confirmed in a validation cohort of 32 CLL cases (cohort B) analyzed by immunophenotyping (Fig. 3A). The cohort analyzed in Fig. 3A included 19 ROR1-low CLL samples and 13 ROR1-high CLL samples. ROR1-high cases had a significantly higher expression levels of Bcl2 than ROR1-low cases (P = 0.007). In summary, the loss of *miR-15/16* expression allows not only for the overexpression of Bcl2 (6) and Mcl1 (25), but also for the overexpression of ROR1.

Incidence of 13q14 Deletion in ROR1-High and ROR1-Low CLL. Since *miR-15/16* target *ROR1*, we investigated for differences in the incidence of 13q14 deletion in ROR1-high versus ROR1-low CLL. Since patients with CLL cells that have a deletion(s) at 13q14 as the sole genetic abnormality have indolent disease, a casual distribution of 13q14 deletion in our set of indolent samples could be expected. Nevertheless, when clustering the samples according to the expression level of ROR1, we found that the distribution of samples harboring a deletion at 13q14 correlated with the expression level of ROR1. Indeed, we examined the CLL cells of 305 patients with indolent disease, finding that in ROR1-high samples, about 53% of cells carry the 13q deletion as opposed to ROR1-low samples in which only about 16% of cells carry the deletion. To further confirm the correlation between the incidence of 13q deletion and ROR1 expression, we examined 2,429 CLL cases form the CLL Research Consortium (CRC) database with assessed ROR1 expression. Of these, 974 cases had 0–10% of cells carrying the 13q deletion and 217 cases had 90–100% of cells carrying the 13q deletion. For samples with more than 90% of 13q-deleted cells, the expression ROR1 was about twofold higher than on samples with less than 10% 13q-deleted cells (Fig. S5). Moreover, the FISH results of the samples included in cohort A revealed that among ROR1-low cases (*n* = 9), one case had CLL cells with trisomy 12, seven cases had normal FISH, and only one had loss of 13q14; in this case, only 17% of CLL cells harbored the 13q14 deletion. In contrast, of the seven ROR1-high cases from the same cohort six (86%) had loss of 13q14 with various percentages of cells carrying the deletion (average 13q deletion percentage = 50.2%) (Table S1). Additional samples used for

Table 1. Differentially expressed microRNAs in ROR1 low and ROR1 high CLL samples

Gene name	ROR1 low	ROR1 high	Linear FC	P value
hsa-miR-199a-5p	62.5	22.1	2.8	0.012
hsa-miR-451a	1,653.0	610.4	2.7	0.006
hsa-miR-151a-3p	71.6	29.6	2.4	0.001
hsa-miR-151a-5p	131.6	55.1	2.4	0.001
hsa-miR-484	33.8	14.6	2.3	0.024
hsa-miR-132-3p	34.0	16.0	2.1	0.030
hsa-miR-199a-3p/ hsa-miR-199b-3p	231.8	116.6	2.0	0.044
hsa-miR-15a-5p	2600.6	1,327.4	2.0	0.006
hsa-miR-365a-3p/ hsa-miR-365b-3p	105.9	59.7	1.7	0.043
hsa-miR-363-3p	183.1	107.1	1.7	0.004
hsa-miR-16-5p	18,347.2	10,848.8	1.7	0.003
hsa-miR-222-3p	1308.6	841.8	1.6	0.004
hsa-miR-337-3p	36.0	56.2	−1.6	0.028
hsa-miR-29a-3p	2,011.4	3,258.2	−1.6	0.002
hsa-miR-664a-3p	213.0	405.2	−1.9	<0.0001
hsa-miR-148a-3p	411.8	1,051.9	−2.6	0.009
hsa-miR-155-5p	1,829.5	5,064.8	−2.8	0.001

A signature of 17 microRNAs can discriminate these two groups of CLL samples. Bold cells show MicroRNAs predicted to target the 3′UTR of *ROR1*.
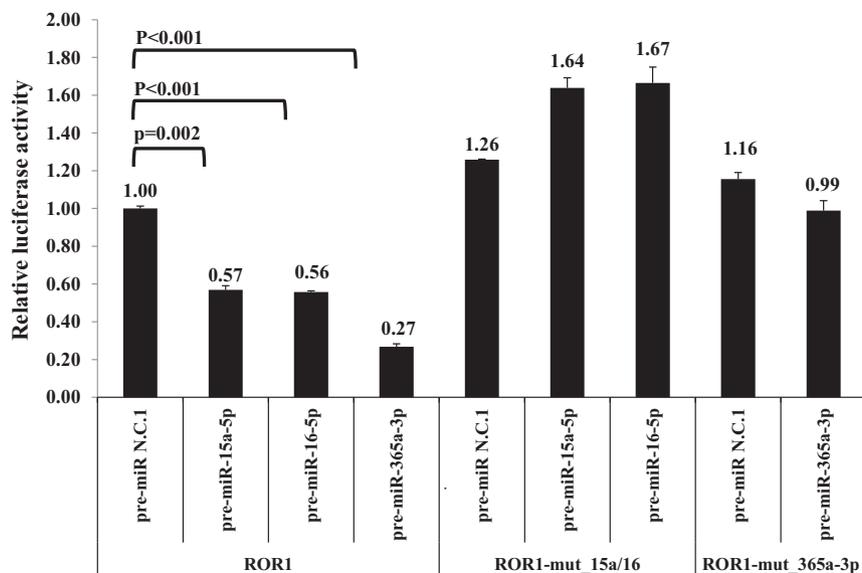
Fig. 1. *MiR-15/16* targets *ROR1* expression. Transfection experiments were performed in HEK-293 cells using constructs indicated. First four bars show results using WT construct. The bars from 5 to 9 show results using constructs with mutant target sites.

Nanostring along with those from cohort A had similar distribution of cytogenetic aberrations. Thus, of the 12 samples with low ROR1 used for Nanostring, 8 (67%) showed a normal karyotype, 2 (17%) carried trisomy 12 aberrations in more than 30% of CLL cells, and 2 (17%) carried 13q14 deletion in less than 17% of CLL cells. Of the 11 samples with high ROR1 for which FISH data were available, 1 (9%) showed a normal karyotype and 10 (91%) had the 13q14 deletion. To confirm these results, we analyzed the relationship between the expression levels of ROR1 and the percentages of CLL cells carrying the 13q14 deletion in cohort B (Fig. 3B) and an additional validation cohort of 256 CLL patients (cohort C) with indolent prognostic markers (Fig. 3C). In cohort B, we found that deletion at 13q14 was more common among ROR1-high CLL than among ROR1-low CLL ($P = 0.001$) (Fig. 3B). Similarly, in cohort C, we observed that CLL cases with high-level expression of ROR1 had a significantly higher percentage of CLL cells carrying the 13q14 deletion than the cases with CLL cells expressing low levels of ROR1 (Fig. 3C) ($P < 0.001$). Table S2 summarizes the phenotype and FISH data of the samples included in each cohort. In summary, high-level expression of ROR1 correlates with the loss of 13q14, where *miR-15/16* are located.

Activity of Cirmtuzumab Alone and in Combination with Venetoclax. Since loss of *miR-15/16* is an early driver in CLL development that may allow for overexpression of both Bcl2 and ROR1, we investigated whether combination therapy with agents targeting each of these proteins would be more effective than either agent alone. To test the ability of cirmtuzumab to kill CLL cells, we analyzed CLL cell viability in the presence of accessory U937 cells. We performed this test with and without venetoclax to evaluate for the synergic activity of these two drugs and the cytotoxic impact of a combination therapy on CLL target cells with a high-level expression of ROR1 (Table S3). After 16 h of treatment, U937 cell viability was not affected by treatment with venetoclax, cirmtuzumab, or control human IgG1 alone or in combination with venetoclax. However, we found that, in the presence of U937 cells, the combination of venetoclax and cirmtuzumab was significantly more cytotoxic than treatment with venetoclax alone, cirtuzumab alone, or in combination with control human IgG1 antibody ($P < 0.01$, Fig. 4). At 16 h, treatment with cirmtuzumab alone did not significantly affect CLL cells viability. Treatment with ABT-199 alone (3 nM) resulted in about 50% of cell death, while addition of

cirmtuzumab resulted in about 75% of cell death, suggesting a synergic effect of the two drugs. Similar results were obtained using 10 nM ABT-199 (Fig. 4A). We previously found that exogenous Wnt5a could enhance the viability, migration, and chemokine-directed migration of ROR1-expressing CLL cells (21). Since U937 cells express Wnt5a (26, 27), we examined whether in vitro treatment of CLL cells with cirmtuzumab alone or in combination with venetoclax could inhibit Wnt5a-induced enhanced viability. For this, CLL cells expressing ROR1 were treated with cirmtuzumab alone or in combination with ABT-199, in the presence of exogenous Wnt5a. After 24 h of treatment, cirmtuzumab alone or in combination with venetoclax could significantly inhibit the Wnt5a-induced enhanced viability (Fig. 4B).

Discussion

The first genetic alteration in the noncoding genome identified in human diseases was the deletion of *miR-15a* and *miR-16-1*, which occurs in ≈70% of CLL cases (4). Therefore, the most common genetic alteration in CLL is the specific deletion of two micro-RNAs that map on the same polycystronic RNA, *miR-15a* and *miR-16-1* (4). *MiR-15/16* expression inhibits cell proliferation by targeting multiple oncogenes and its down-regulation has been reported not only in CLL but also in solid tumors (4, 28) such as non-small cell lung cancers, pituitary adenomas, and prostate carcinomas (29–31). Prediction analysis indicating that the 3′UTR of *BCL2* could be a target of *miR-15/16* was previously validated (6). Thus, loss of *miR-15a* and *miR-16-1* leads to overexpression of Bcl2, which then acts as a driver for malignant transformation (6, 32). A consequence of this finding is that CLL cells are sensitive to treatment with inhibitors of Bcl2. The initial work of Fesik and coworkers led to the development of ABT-199 (venetoclax), which specifically inhibits Bcl2. This compound can induce tumor lysis and complete or partial remissions in most CLL patients without apparent long-term adverse effects (11–14), supporting the findings with *Bcl2* knock-out mice (13). A major problem with effective targeted therapy for CLL is that when a patient has a large number of leukemia cells, it is likely that few mutant cells may be resistant to the effect(s) of the drug of choice, resulting in selection of cells that are refractory to the drug (25, 33). One way to address this problem is to identify other targetable markers that are generated by the same genetic alteration that drives the malignancy. For this reason, we looked at the dysregulation of other
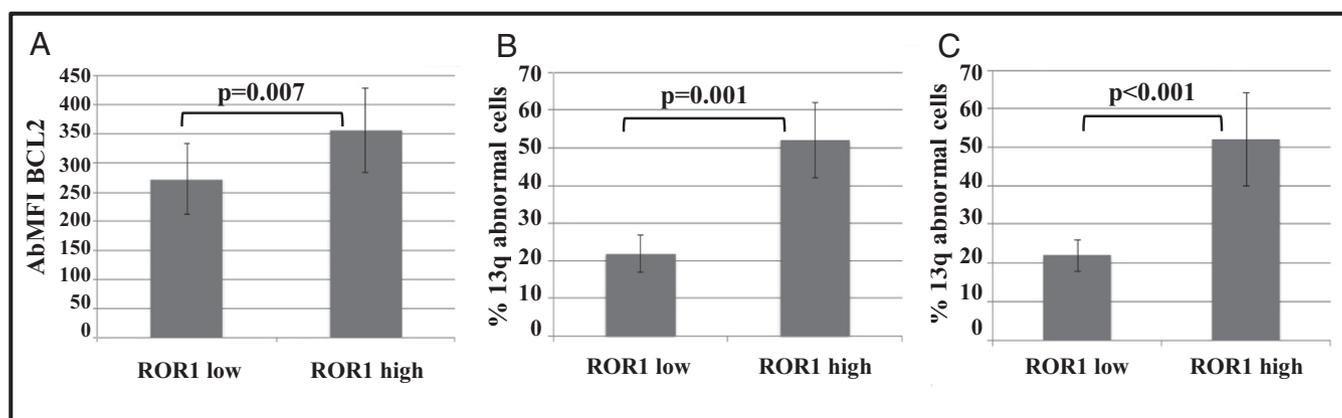
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Fig. 2. Expression of *miR15/16*, ROR1, and Bcl2 in CLL. (*A* and *B*) Reverse correlation of *miR15a* and *miR16-1-5p* with ROR1 expression in CLL. (*C*) Graphic representation of data in *A* and *B*. (*D*) Correlation between Bcl2 and ROR1 expression. Jurkat cells were used as a positive control for Bcl2, and Raji cells were used as negative control for Bcl2. (*E*) Densitometry analysis of data in *D*. The Mann–Whitney *u* test was used to calculate *P* values. (*F*) Correlation between Bcl2 and ROR1 expression in CLL samples used in *D*. (*G*) Correlation between Bcl2 and ROR1 expression, measured by flow cytometry, in all CLL samples included in cohort A. Intracellular Bcl2 (AbMFI) is plotted on the y axis, and the AbMFI of surface ROR1 is charted on the x axis. (*H*) Graphic representation of data in *G*.

gene products targeted by *miR-15/16* and found that ROR1, an onco-embryonic antigen expressed on the majority of CLL cases (17), could be a possible candidate (21). In this study, we found that *ROR1* (as *BCL2*) is a target of *miR-15/16*. Screening of a large collection of CLL samples indicated that over 90% of CLL cases have B cells expressing detectable ROR1 (21). The finding that *ROR1* is regulated by *miR-15/16* suggests that ROR1 represents an additional target for CLL therapy, and a possible supplementary target for combination therapy to reduce the risk of drug resistance and to enhance the response to treatment with venetoclax. Indeed, the cases with the highest levels of ROR1 may be relatively resistant to venetoclax because of the associated high-level expression of Bcl2. Also, each drug would affect the same cells by targeting two different genes regulated by the same microRNAs lost in the leukemia clone. Thus, the selection of variants resistant to both therapy agents would be unlikely. To determine if venetoclax can be used in combination with cirmtuzumab, we treated CLL cells from three different patients (Table S3) with venetoclax and/or cirmtuzumab, in the presence of U937

accessory cells; we observed a twofold drop in CLL viability in the cells exposed to a combined treatment compared with CLL cells treated with either agent alone (Fig. 4*A*), indicating that a combination therapy could improve the response to treatment. In addition, we previously found that the viability of ROR1-expressing CLL cells could be enhanced by Wnt5a (21), which is expressed by U937 cells (26, 27). Here, we find that Wnt5a could enhance the resistance of CLL cells to venetoclax. However, treatment of CLL cells with cirmtuzumab could block the capacity of exogenous Wnt5a to mitigate the cytotoxic activity of venetoclcax for CLL cells that expressed ROR1.

Most patients with indolent disease have B cells with deletions at 13q14 as a sole cytogenetic abnormality. However, CLL cells with 13q deletion can progress by developing additional chromosomal alterations. We selected our samples considering that most indolent CLLs do not bear additional chromosomal abnormalities. Our study of indolent samples that vary in their expression levels of ROR1 revealed that the distribution of CLL carrying a 13q deletion is not random, but correlates with the

Fig. 3. Relationship among ROR1, Bcl2, and percentage of CLL B cells harboring a 13q deletion. Cohort B (*n* = 32) was examined for Bcl2 expression and percentage of CLL cells carrying 13q deletion by FISH analysis. (*A*) Bcl2 expression in ROR1-low and ROR1-high CLL samples. (*B*) Percentage of 13q-deleted cells in ROR1-low and ROR1-high CLL samples from cohort B. Samples with low expression of ROR1 had an average expression of intracellular Bcl2 of AbMFI = 272 (*A*) and an average percentage of CLL B cells carrying a 13q deletion of 22.3% (*B*). CLL cases with high expression of ROR1 had a mean expression of Bcl2 AbMFI = 356 (*A*) and had an average percentage of CLL B cells with a 13q deletion of 55.2% (*B*). (*C*) Percentage of 13q-deleted cells in ROR1-low and ROR1-high CLL samples in cohort C (*n* = 256). CLL samples with low expression of ROR1 had an average of 20.8% of CLL cells carrying 13q deletion. CLL cases with high expression of ROR1 had an average 52% of CLL B cells with 13q deletion (Table S2).

expression level of ROR1. Thus, CLL cells with high-level expression of ROR1 carry a deletion of chromosome 13q, while CLL cells with low-level expression of ROR1 have either a normal karyotype or a trisomy of chromosome 12. Furthermore, since the 13q14 chromosome deletion is associated with loss of *miR-15/16* (4, 5), which also target *BCL2* (6), we would expect that CLL cases with high-level expression of ROR1 and Bcl2 also would have a higher percentage of CLL B cells carrying the 13q14 deletion. Our studies indicate that this is indeed the case (Fig. 3 *B* and *C*). These are important findings that can be applied to all CLL patients, regardless of the clinical course of the disease. Indeed, the 13q14 deletion can be detected by cytogenetics (FISH) in 55% of cases, and small deletions affecting the *miR-15/16* gene are observed in a large fraction of CLL samples (4). Consequently, up to 70% of CLL cases lose or down-regulate expression of *miR-15/16* (4). Since over 90% of CLL cases express ROR1, in the minority of cases when CLL is lacking deletion at 13q14, other mechanisms may be involved in the

down-regulation of *miR-15/16*. Indeed, our group demonstrated that p53 is a positive activator of *miR-15/16* (34), and p53 is deleted in 7–10% of CLL samples. Thus, loss of p53 may lead to down-regulation of *miR-15/16* (34) in CLL-harboring deletions in 17p (2), which generally confers resistance to chemotherapy because of its association with the loss of functional p53 (2). This mechanism also explains why the 13q deletion is a good prognostic factor, while high-level expression of ROR1 is a poor prognostic factor. Indeed, during CLL progression, additional chromosomal aberrations can affect the levels of expression of Bcl2 and ROR1, and 13q deletion is a good prognostic only when found as the sole cytogenetic abnormality. Many aggressive CLLs have additional chromosomal abnormalities (to 13q deletion), resulting in dysregulation of additional pathways where overexpression of Bcl2 and ROR1 promote CLL cell proliferation and resistance to apoptosis.

The discovery that *miR-15/16* target *ROR1* and *BCL2* may lead to the development of effective combination therapies that target
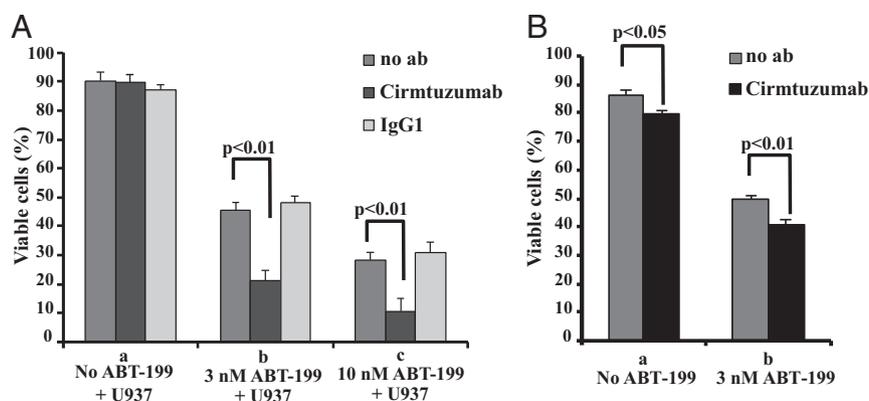

Fig. 4. CLL cell viability with venetoclax and cirmtuzumab. (*A*) Bars indicate the average percentage of viable CLL cells assessed after 16-h treatment in presence of U937 accessory cells. (*a*) Cirmtuzumab treatment: cells treated with no antibody (dark gray bar), with 20 μg/mL cirmtuzumab alone (black bar), or with 20 μg/mL human IgG1 antibody (light gray bar). (*b*) Venetoclax-cirmtuzumab combination 1: cells were treated with 3 nM venetoclax alone (dark gray bar), or in combination with 20 μg/mL cirmtuzumab (black bar) or human IgG1 antibody (light gray bar). (*c*) Venetoclax-cirmtuzumab combination 2: cells were treated with 10 nM venetoclax with no antibody (dark gray bar), or in combination with 20 μg/mL cirmtuzumab (black bar) or human IgG1 antibody (light gray bar). U937, human monocyte cell line; Cirmtuzumab, anti-human *ROR1* antibody; IgG1, anti-human IgG1 antibody; No Ab, no antibody control. (*B*) CLL cells expressing ROR1 were cultured in serum-free media and treated with cirmtuzumab (20 μg/mL) alone or in combination with ABT-199 (3 nM), in the presence of exogenous Wnt5a (200 ng/mL). Bars indicate the average percentage of viable CLL cells assessed after 24 h of treatment. Data are shown as mean ± SEM.

the same leukemia cells through different biomarkers. ROR1 is a surface antigen that can be targeted with antibodies (17), and antibodies against ROR1 already have been generated (24). A phase 1 clinical trial of cirmtuzumab demonstrated that, as a single agent, cirmutuzumab is safe and well tolerated and has biologic activity and specific target inhibition (24), since virtually all normal adult tissues lack expression of ROR1. Combination therapy of venetoclax and cirmtuzumab may have additive effects in eradicating CLL cells and mitigate the risk of clonal selection that could lead to resistance to either of these drugs alone (33). We believe that this is an important finding, which may help potentially cure patients with CLL: miR-15/16 simultaneously affects two key genes (BCL2 and ROR1) in development and progression of CLL. Additionally, these functional drugs that target the proteins encoded by these genes may be used in combination to improve the response to treatment over that achieved with either agent alone, without incurring additional toxicity, potentially providing for highly effective therapy of patients with this disease.

Methods

Study Design and Participants. For this study, we enrolled CLL patients who provided written, informed consent, in compliance with the Declaration of Helsinki and the Institutional Review Board of the University of California at San Diego and the Ohio State University. Blood was obtained from 305 CLL patients enrolled in the CRC who satisfied diagnostic and immunophenotypic criteria for CLL. These patients were grouped into three cohorts (cohort A: $n =$ 17, cohort B: $n = 32$, cohort C: $n = 256$). All of the $n = 305$ samples were randomly chosen from patients with indolent prognostic markers (IGHV mutated and ZAP-70 negative) who differed by in their expression of ROR1. All samples were collected from not-treated patients and within 3 y from CLL

diagnosis (Table S2). Based on the threshold previously identified (21), leukemic cells with an average absolute mean fluorescence intensity (Ave AbMFI) of less than 10 were defined as ROR1-low, and those with an average AbMFI greater than 40 were defined as ROR1-high (Table S2). Samples were selected to have similar number of cases expressing high or low levels of ROR1, so that the comparison would be significant.

Patients Cohorts. Cohort A comprises 17 CLL samples were initially used as a test cohort for miR profiling and for Bcl2 expression level assessment by flow cytometry and immunoblot. Of these, nine patients show low levels of ROR1 (Ave AbMFI = 5.7) and eight show high levels of ROR1 (Ave AbMFI = 53.0) (Tables S1 and S2). Cohort B comprises 32 CLL samples were used as validation cohort for assessment of Bcl2 expression by flow cytometry. Of these, 19 samples expressed low levels of ROR1 (Ave AbMFI = 5.1) and 13 expressed high levels of ROR1 (Ave AbMFI = 79.3) (Table S2). Two samples from cohort B, both ROR1-high cases, were also used for miR profiling (Table S1). Cohort C comprises 256 CLL samples with B cells carrying various amounts of the 13q deletion were additionally used to establish the correlation between ROR1 expression and the percentage of CLL cells carrying the 13q deletion (ROR1-low cases $n =$ 26 with an Ave AbMFI = 5.3, ROR1-high cases $n = 230$ with an Ave AbMFI = 40.1) (Table S2). Five samples from cohort C were also used for miR profiling. Of these, three were ROR1-low cases and two were ROR1-high cases (Table S1).

See additional methods in SI Methods.

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