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Original Article

Transient resistance to DNA damaging agents is associated with expression of microRNAs-135b and -196b in human leukemia cell lines

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Abstract: The acquisition of resistance to anticancer drugs is widely viewed as a key obstacle to successful cancer therapy. However, detailed knowledge of the initial molecular events in the response of cancer cells to these chemotherapeutic and stress responses, and how these lead to the development of chemoresistance, remains incompletely understood. Using microRNA array and washout and rechallenge experiments, we found that short term treatment of leukemia cells with etoposide led a few days later to transient resistance that was associated with a corresponding transient increase in expression of ABCB1 mRNA, as well as microRNA (miR)-135b and miR-196b. This phenomenon was associated with short-term exposure to genotoxic agents, such as etoposide, topotecan, doxorubicin and ionizing radiation, but not agents that do not directly damage DNA. Further, this appeared to be histiotype-specific, and was seen in leukemic cells, but not in cell lines derived from solid tumors. Treatment of leukemic cells with either 5-aza-deoxycytidine or tricostatin A produced similar increased expression of ABCB1, miR-135b, and miR-196b, suggesting a role for epigenetic regulation of this phenomenon. Bioinformatics analyses revealed that CACNA1E, ARHGEF2, PTK2, SIAH1, ARHGAP6, and NME4 may be involved in the initial events in the development of drug resistance following the upregulation of ABCB1, miR-135b and miR-196b. In summary, we report herein that short-term exposure of cells to DNA damaging agents leads to transient drug resistance, which is associated with elevations in ABCB1, miR-135b and miR-196b, and suggests novel components that may be involved in the development of anticancer drug resistance.

Keywords: Genotoxic agents, microRNAs, miR-135b, miR-196b, transient anticancer drug resistance

Introduction

Although cancer is one of the leading causes of death worldwide [1], chemotherapy has improved overall survival and quality of life for patients with many cancers. However, the development of multidrug resistance (MDR) is widely regarded as a major impediment to effective chemotherapy [2]. At present, MDR is considered to be a multifactorial phenomenon associated with various mechanisms, including changes in the level of protein targets, altered metabolism of drugs, and reduced intracellular drug accumulation [3, 4]. Cell lines expressing MDR are typically isolated by multiple selections, in which cells become adapted to growth in progressively higher concentrations of the selecting drug [5]. A number of reports have pointed to an acute response in the increased expression of the multidrug resistance gene (ABCB1) and its protein product (P-glycoprotein, P-gp), a transmembrane ATP-dependent transporter molecule, after cells were treated with different xenobiotics such as differentiating agents [6], antineoplastic drugs [7, 8], or other stressors, such as heat shock [9]. In addition, it has been reported that transient exposure to anticancer drugs induces ABCB1 mRNA expression in subpopulations of treated cells, and drug-induced resistance was sustained for 6 weeks after the removal of the drug [10]. However, expression of P-gp is by no means the only mechanism of MDR in clinical cancers, and simply overcoming or circumventing its activity

would not be expected to cure all MDR-associated cancers [11]. Indeed, clinical studies designed to circumvent P-gp-associated MDR have been unsuccessful [12, 13]. In another study, 'drug-tolerant persisters' re-sensitized to treatment following drug withdrawal suggested a reversible chromatin-mediated dynamic regulation [14], followed by an equation model demonstrating transient emergence of drug resistance as a result of phenotypic fluctuations and selection pressure [15]. More recently, a kind of specific transient resistance to small molecule tubulin binding diaminothiazoles was reported [16]. Additionally, a 'poised' epigenetic state, in which cells are reversibly drug resistant but can acquire a stable resistant phenotype on continued drug exposure, has been proposed [17]. However, detailed knowledge of the initial molecular events in the response of cancer cells to these chemotherapeutic and stress responses, and how these responses are connected to the development of chemoresistance remains elusive. Moreover, targeting any single molecule appears to be insufficient to reverse chemotherapeutic resistance, indicating that multiple molecular pathways may contribute to the sensitivity of cancer cells to chemotherapy [18]. Accordingly, our goal here was to understand the ability of cancer cells to adapt rapidly to the selective pressures brought about by their treatment with chemotherapeutic agents.

microRNAs (miRNAs) are small, noncoding RNAs that usually act as posttranscriptional repressors of gene expression by binding to the 3'-untranslated region (3'-UTR) of their targets [19]. Accounting for about 3% of human genes [20], miRNAs are predicted to regulate approximately 30% of human proteins through effects on mRNA expression, depending on the degree of complementarity between the miRNA and its target [21]. Considerable evidence suggests that these small RNAs are implicated as important effectors in the pathogenesis of cancer as well as response to treatment [22, 23]. In addition, compared to normal tissues, altered expression profiles of miRNAs have been shown in various tumors including different types of leukemias [24]. Therefore, the breadth of regulatory effects mediated by miRNAs has led to the hypothesis that aberrant expression of miRNA contributes to MDR [25]. However, the precise contribution of miRNAs to drug responsiveness and anticancer resistance and the mechanisms underlying their dysregulation remain largely unexplored.

In the present study, we examined the involvement of miR-135b and miR-196b in response to xenobiotic stressors in drug-sensitive and -resistant human leukemic cell lines and attempted to assess the possible associations of miRNAs and the emergence of anticancer drug resistance under conditions that traditionally select for the development of drug resistant tumor cells.

Materials and methods

Cell lines and culture conditions

The human T-cell leukemic cell line CCRF-CEM, its teniposide (VM-26)-resistant subline, CEM/VM-1-5, the human rhabdomyosarcoma cell line Rh30 and its etoposide (VP-16)-resistant subline, Rh30/V1, described previously [5, 26, 27], were grown in RPMI 1640 supplemented with 10% FBS. The human breast cancer cell line MCF7 (MCF7/WT) and its VP-16-resistant subline (MCF7/VP) were kindly provided by Dr. E. Schneider (Albany, NY) [28] and grown in DMEM containing 10% FBS. Jurkat cells were kindly provided by Dr. David Ucker (University of Illinois at Chicago) and grown in RPMI-1640 with 2 mM L-glutamine, 10% FBS, and 0.5 μM 2-mercaptoethanol. RPMI 8226, HL-60 and MOLT-4 were obtained from American Type Culture Collection (Manassas, VA) and maintained, respectively, in RPMI-1640 with 10% FBS and 0.5 μM 2-mercaptoethanol; RPMI 1640 supplemented with 10% FBS; IMDM supplemented with 20% FBS; and RPMI 1640 supplemented with 10% FBS. Ovarian cancer A2780 cells were obtained from the National Cancer Institute and were maintained in DMEM supplemented with 10% FBS.

Profiling of miRNA expression

Global profiling of miRNA expression of CCRF-CEM and CEM/VM-1-5 cells was performed using the Human MicroRNA Array v1.0 (Applied Biosystems Inc, Darmstadt, Germany), which contains 365 human miRNAs and two small nucleolar RNAs (snoRNAs) that function as endogenous controls. The relative amount of each miRNA was normalized to RNU48 snoRNA. The fold-change for each miRNA was calculated by the comparative $2^{-\Delta\Delta CT}$ method [29].

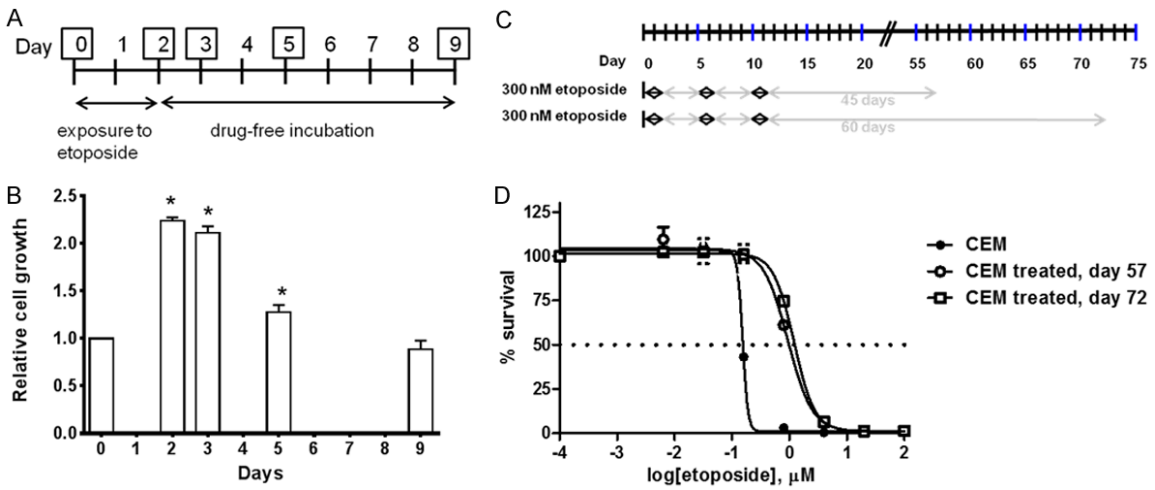

Figure 1. Drug resistant phenotype changes after short-term chemotherapeutic drug exposure. A. Schematic of the washout experimental design. B. Treatment of CCRF-CEM cells with 300 nM of etoposide for 48 h resulted in subsequent increases in number of surviving cells. The apparent acquired transient drug resistance gradually decreased starting at day 3 (24 h after etoposide removal), day 5 (72 h after etoposide removal) and day 9 (7 d after etoposide removal). Relative cell growth was used to determine the resistance of CCRF-CEM cells to etoposide at indicated time points. See Figure S1 for experimental details. C. Schematic of the rechallenge experimental design. D. Drug sensitivity assay determined that the IC_{50}-value increased from 300 nM to 1 μM in CCRF-CEM cells exposed to repeated drug challenge after either 15 or 20 passages in drug-free medium. Values are mean ± SE (n = 3). *, $p < 0.05$.

TaqMan quantitative real-time polymerase chain reaction (qRT-PCR)

TaqMan individual real-time RT-PCR miRNA kit (Applied Biosystems Inc) including RT primers and TaqMan probes were used to quantify the expression levels of mature miRNA-135b (AB: 4373139), miRNA-146b (AB: 4373178), miRNA-196b (AB: 4373103), and miRNA-615 (AB: 4380991) in cell lines. Normalization was performed with small nucleolar RNA, RNU48 (AB: 4373383). miRNA expression levels were quantified with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc). Relative expression was calculated by the comparative $2^{-\Delta\Delta CT}$ method [29].

Radiation sensitivity assay

Irradiation was done by exposing cells to X-rays produced by a Varian 21EX linac with an energy of 6 MV (Varian Medical Systems, Palo Alto, CA) at a dose rate of 1 Gy/min. Cells were irradiated to the total dose called for in the design of each experiment. The radiation doses used in these experiments were chosen to cover a wide range: 0, 0.5, 1, 2, and 6 Gy. Cells were then seeded in 96-well plates in growth medium following varying doses of radiation and incubated at 37°C for 4 days. Cell viability was assessed using the CellTiter 96 AQueous assay according to the manufacturer's instructions (Promega, Madison, WI).

Transduction

The plasmid expressing either miR-135b or miR-196b in lentiviral vector pCDH-CMV-MCS-EF1-copGFP (CD511B-1) was from System Biosciences (Mountain View, CA). Packaging and infection of cells was done according to the manufacturer's protocol.

Cell viability assay

Cells were seeded in 96-well plates at a density of 2×10^3 cells per well and grown overnight. The cells were then treated with drugs at varying concentrations (0, 0.0064, 0.032, 0.16, 0.8, 4, or 20 μM of etoposide) and incubated at 37°C for 4 days. % survival of cells was determined by relative cell growth in response to varying concentrations of etoposide compared to vehicle (DMSO) treatment. Cell viability was assessed using the CellTiter 96 AQueous assays (MTS assays) according to the manufacturer's instructions (Promega, Madison, WI).

Gene ontology (GO) analyses

Gene Ontology (GO) analyses were performed with the web tool DAVID at http://david.abcc.

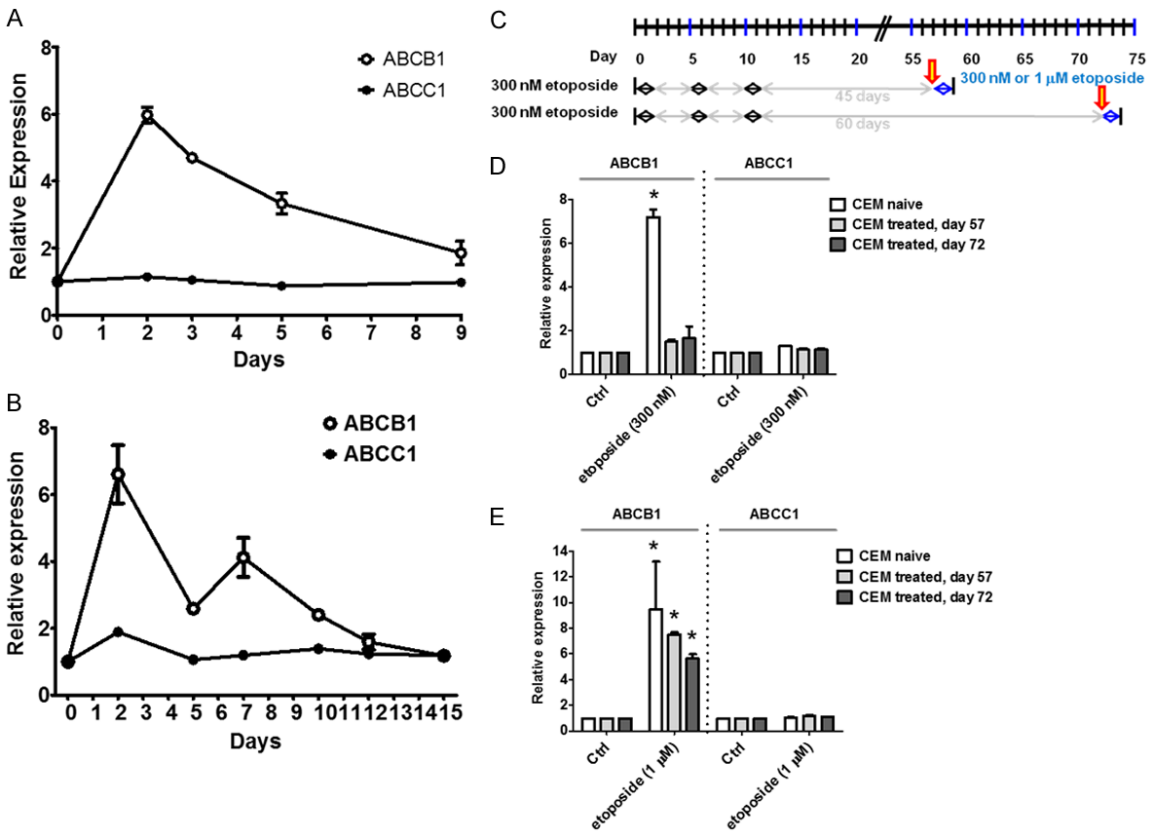

Figure 2. Elevated expression of ABCB1 after short-term chemotherapeutic drug exposure is associated with transient drug resistance. A. Exposure of CCRF-CEM cells to etoposide for 48 h led to upregulation of ABCB1 expression, but not ABCC1, which gradually decreased after withdrawal of etoposide. B. Subsequent etoposide (IC_{50}, 300 nM) rechallenges (2nd and 3rd challenge, days 5 and 10) led to progressively attenuated expression of the ABCB1 gene compared to its expression after the first challenge using the same etoposide concentration. C. Scheme of the rechallenge experimental design. Cells were exposed to 300 nM etoposide for 48 h 3 times, with a 3-day drug-free incubation in between. Cells were then incubated in drug-free medium for either 15 or 20 passages and exposed to 300 nM etoposide for 48 h. D. Treatment of these previously-exposed cells again with 300 nM etoposide resulted in attenuation of ABCB1 induction response whereas there was no change in ABCC1 expression. E. Treatment of these previously-exposed cells again with 1 μM etoposide led to induction of ABCB1 expression whereas there was no change in ABCC1 expression. Naïve CCRF-CEM cells were used as positive control. Values are mean ± SE (n = 3). *, $p < 0.05$.

ncifcrf.gov/home.jsp using default parameters [30]. GO analyses were performed for predicted targets of miR-135b and miR-196b, respectively. After performing the analysis, we retained only biological process (BP)/molecular function (MF)/cellular component (CC) categories with a p-value ≤ 0.001, FDR ≤ 5, and fold enrichment ≥ 2 in the analysis, and we eliminated redundant terms and noninformative terms (e.g., multigene family).

Statistical analyses

Each experiment was repeated at least three times. Numerical data are presented as mean ± SE. Comparisons between groups were analyzed using the Student's *t*-test (two groups) or a one-way ANOVA followed by post hoc Tukey test (multiple groups). Differences with *P* values less than 0.05 are considered significant.

Results

Drug resistant phenotype changes after short-term chemotherapeutic drug exposure

To understand the ability of cancer cells to adapt to the selective pressures brought about by their treatment with chemotherapeutic agents, we studied the initial events in the development of drug resistance in response to chemotherapeutic challenge. As shown in

Figure 1A, we conducted washout experiments to measure the cell viability at various time points by MTS assay. We first measured baseline cell viability in control CCRF-CEM cells before adding etoposide to the culture medium (Day 0). We then incubated CCRF-CEM cells with 300 nM etoposide (IC_{50}) for 48 h (Day 2). Etoposide was then removed from the medium, after which the CCRF-CEM cells were incubated in etoposide-free medium for up to 7 d. Cell viability was monitored continuously after the removal of etoposide at day 3 (24 h after etoposide removal), day 5 (72 h after etoposide removal) and day 9 (7 d after etoposide removal) and was assessed by MTS assay to determine the relative resistance of CCRF-CEM cells to etoposide. Cells from above time points were subjected to MTS assays (Figure S1). We found that increased cell growth (drug resistance) in CCRF-CEM cells correlates with the presence of drug (**Fiure 1B**). In addition, this apparent "acquired drug resistance" decreased with increased time of incubation of the cells in drug-free medium, as the cells returned to baseline sensitivity by day 9.

To investigate the kinetics of changes in the drug resistance phenotype, we performed rechallenge experiments in which CCRF-CEM cells were repeatedly exposed to etoposide for 48 h with a 3-day drug-free incubation in between and then incubated in drug-free medium for either 15 or 20 passages; experimental design is shown in **Figure 1C**. We asked whether this chemotherapeutic rechallenge can lead to drug resistance. To this end, we measured the etoposide IC_{50} in CCRF-CEM cells exposed to repeated drug challenge after either 15 or 20 passages in drug-free medium (at day 57 and day 72, respectively). We found that the etoposide IC_{50} in these cells had increased from 300 nM to 1 μM (**Figure 1D**), indicating that the etoposide IC_{50} is increased and these cells are (stably) drug-resistant. Our results suggest that the acquired drug-resistance phenotype seems to re-set the IC_{50} to higher levels.

Elevated expression of ABCB1 after short-term chemotherapeutic drug exposure is associated with transient drug resistance

To better understand the mechanism for the establishment of the drug-resistance phenotype, we investigated the expression of ABCB1 (P-gp), whose upregulation is seen following DNA damage [31], and ABCC1 (MRP1), two of the most extensively characterized transporters associated with MDR [32]. As shown in **Figure 2A**, exposure of CCRF-CEM cells to etoposide for 48 h resulted in increased ABCB1 expression that gradually decreased after withdrawal of etoposide. By contrast, we observed no significant changes in ABCC1 expression under these conditions. Our findings suggest that the transient expression of ABCB1 is associated with acquired drug resistance.

To investigate the kinetics of expression of ABCB1 and ABCC1, we performed re-challenge experiments in CCRF-CEM cells as described above (**Figure 1C**). We found changes in the expression of the ABCB1 gene, but not the ABCC1 transporter gene following short-term etoposide exposure, consistent with the fact that ABCC1 is regulated differently from ABCB1 [33]. In addition, subsequent etoposide (IC_{50}, 300 nM) re-challenges (2^{nd} and 3^{rd} challenge, days 5 and 10) led to progressively attenuated expression of the ABCB1 gene compared to its expression after the first challenge using the same etoposide concentration (**Figure 2B**). These results suggest that ABCB1 is associated with the initial response of cancer cells to chemotherapeutic challenge, but less so with subsequent drug challenges, suggesting that mechanisms other than ABCB1 expression are involved in this progressive drug resistance. We next asked whether this expression of ABC transporters is associated with the altered drug responsiveness caused by chemotherapeutic rechallenge. To this end, we measured expressions of ABCB1 and ABCC1 in CCRF-CEM cells exposed to repeated drug challenge after either 15 or 20 passages in drug-free medium. Treatment of these previously-exposed cells again with 300 nM etoposide resulted in unexpected attenuation of ABCB1 expression (**Figure 2C**, **2D**). As before, we saw no changes in expression of ABCC1. Next, we asked whether these resistant cells are still responsive to drug at a higher concentration. For this, we treated these previously-exposed cells with 1 μM etoposide (new IC_{50} determined in **Figure 1D**), and measured expressions of ABCB1 and ABCC1. Indeed, when we used this treatment, the expression of ABCB1 was induced as expected (**Figure 2E**), suggesting

Table 1. Validation of miRNAs differentially expressed in CEM/VM-1-5 cells compared to CCRF-CEM cells

microRNAs	Fold change (CEM/VM-1-5/CEM)
hsa-miR-135b	44.5 ± 8.7
hsa-miR-146b	30.5 ± 9.6
hsa-miR-196b	102.4 ± 35.6
hsa-miR-615	6.8 ± 1.6
hsa-miR-345	0.5 ± 0.1

Real-time RT-PCR analysis was carried out to validate the microarray results. Triplicate assays were performed for each RNA sample and the relative amount of each miRNA was normalized to RNU48 snoRNA. Data is shown as fold changes of miRNA levels in CEM/VM-1-5 cell line relative to CEM cell line, which is set as 1 (means ± SE).

that the drug resistant state becomes stabilized over time and the changes seen are quantitative rather than qualitative. The results also suggest that ABCB1 may be involved in the development of drug resistance. The attenuation of response is consistent with the development of acquired resistance during the course of treatment, in which tumors that are not initially resistant to a particular drug develop resistance quickly. The acquisition of resistance to a broad range of anticancer drugs may be due to prevailing selection and overgrowth of drug-resistant variants with many genetic changes, resulting in the futility of the treatment. It seemed to us that the expression of ABCB1 is just one marker of drug resistance, so we sought other markers that might reflect these pleiotropic effects. Accordingly, we examined the expression of miRNAs in these leukemic cells and attempted to assess the possible associations of miRNAs and anticancer drug resistance.

MiRNAs are differentially expressed in a multidrug resistant human T-cell leukemia cell line compared to controls

To examine the involvement of miRNAs in MDR, we profiled the expression of 365 miRNAs in drug-sensitive CCRF-CEM human T-cell leukemia cells and the multidrug resistant CEM/VM-1-5 cells (selected for resistance to teniposide, also cross-resistance to etoposide) using TaqMan™ Human MicroRNA Array v1.0. Based on independent triplicate experiments, combined with validation using qRT-PCR, we confirmed four miRNAs as upregulated and one as downregulated in CEM/VM-1-5 cells, compared to CCRF-CEM cells (**Table 1**). We did not observe the same expression pattern of those validated miRNAs in two other cell lines and their drug-resistant derivatives: breast cancer cell line MCF7/MCF7/VP, and rhabdomyosarcoma cell line Rh30/Rh30/V1, suggesting a context-dependent pattern for miRNA expression. Among these differentially-expressed miRNAs in CEM/VM-1-5 cells, the upregulation of miR-135b and miR-196b expression was among the most pronounced. Accordingly, we focused on these two miRNAs in our subsequent experiments.

Upregulation of miR-135b and -196b following short-term exposure to genotoxic agents in a time- and dose-dependent manner in leukemic cell lines

To examine whether miR-135b and miR-196b have a role in or are associated with the development of drug resistance, we exposed CCRF-CEM cells to 300 nM etoposide (IC_{50}), and measured the expression of miR-135b and miR-196b at various time points by qRT-PCR. Since we observed cell death after 48 h incubation with etoposide, we assayed miR-135b and -196b up to 48 h to avoid measuring cell death events and to focus on short-term selection effects. Expression of miR-135b and miR-196b began to increase after 24 h exposure to etoposide, a DNA damaging agent (**Figure 3A**). In contrast, we saw no substantial increase in expression of either miR-135b or miR-196b in cells treated with vinblastine, a microtubule assembly inhibitor (**Figure 3B**). These results suggest that the upregulation of miR-135b and miR-196b may be a consequence of DNA damage. To further study the dose-response effect of chemotherapeutic drugs on miR-135b and miR-196b expression, we assessed the expression of miR-135b and miR-196b in CCRF-CEM cells treated with varying concentrations of different chemotherapeutic drugs for 48 h. Expression of miR-135b and miR-196b increased in a dose-dependent fashion in response to etoposide (**Figure 3C**). As shown, we observed the same phenomenon after treatment of cells with a topoisomerase I inhibitor (topotecan) (**Figure 3D**), and another topoisomerase II inhibitor (doxorubicin) (**Figure 3E**) at indicated concentrations. In contrast, no significant changes were seen when CCRF-CEM cells were treated with a microtubule assembly inhibitor (vinblastine) (**Figure 3F**) or a microtu-

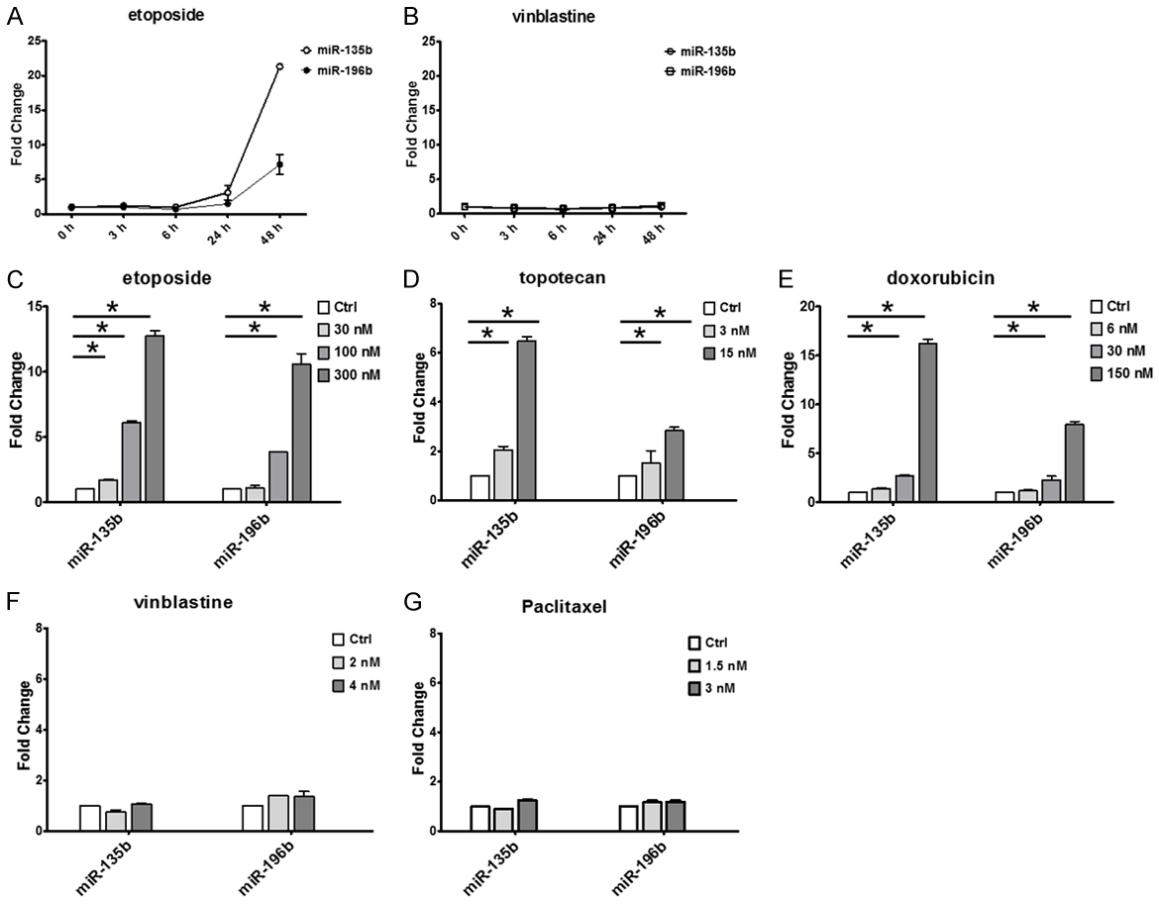

Figure 3. miR-135b and miR-196b are upregulated in response to DNA damaging drugs in a time- and dose-dependent manner in CCRF-CEM cells. (A) miR-135b and -196b expression began to increase after 24 h exposure to 300 nM of etoposide, a DNA damaging agent (IC_{50}: 300 nM). (B) In contrast, no substantial increase in miR-135b and -196b expression in cells treated with 4 nM of vinblastine, a microtubule assembly inhibitor (IC_{50}: 4 nM). CCRF-CEM cells were then exposed to DNA damaging agents (etoposide, topotecan, and doxorubicin) or non-DNA damaging agents (vinblastine and paclitaxel) at the indicated concentrations. Expressions of miR-135b and miR-196b increased in a dose-dependent fashion after treatment of cells with (C) etoposide (IC_{50}: 300 nM), (D) topotecan (IC_{50}: 15 nM), and (E) doxorubicin (IC_{50}: 150 nM) at different concentrations. In contrast, no significant changes were seen when CCRF-CEM cells were treated with (F) vinblastine (IC_{50}: 4 nM) or (G) paclitaxel (IC_{50}: 3 nM). Total RNA was collected after 48 hr of drug incubation. Values are mean ± SE (n = 3). *, $p < 0.05$.

bule stabilizer (paclitaxel) (**Figure 3G**), further implying that the induction of miR-135b and miR-196b may be a consequence of DNA damage.

DNA strand breaks induced by radiation have been quantitated in human leukemia CCRF-CEM cell line using the Comet assay (single-cell gel electrophoresis) [34]. To confirm that DNA damage is involved in this phenomenon, we first measured the dose-response effect of ionizing radiation on CCRF-CEM cells at doses ranging from 0 to 6 Gy, revealing an ID_{50} for CCRF-CEM cells of 2 Gy (**Figure 4A**). When we treated CCRF-CEM cells with ionizing radiation at the indicated doses up to 2 Gy, miR-135b and miR-196b were both upregulated in a dose-dependent manner (**Figure 4B**). We further evaluated expression of miR-135b and miR-196b in CCRF-CEM cells in response to ionizing radiation with respect to time. We performed RT-PCR using cells collected at several time points after radiation exposure ranging from 0 h to 48 h. These experiments demonstrated that the elevation of miR-135b and miR-196b correlated positively with the time after radiation exposure (**Figure 4C**, **4D**), and suggest that this phenomenon is a consequence of DNA damage.

Our experiments raised the question whether increased expression of miR-135b and miR-

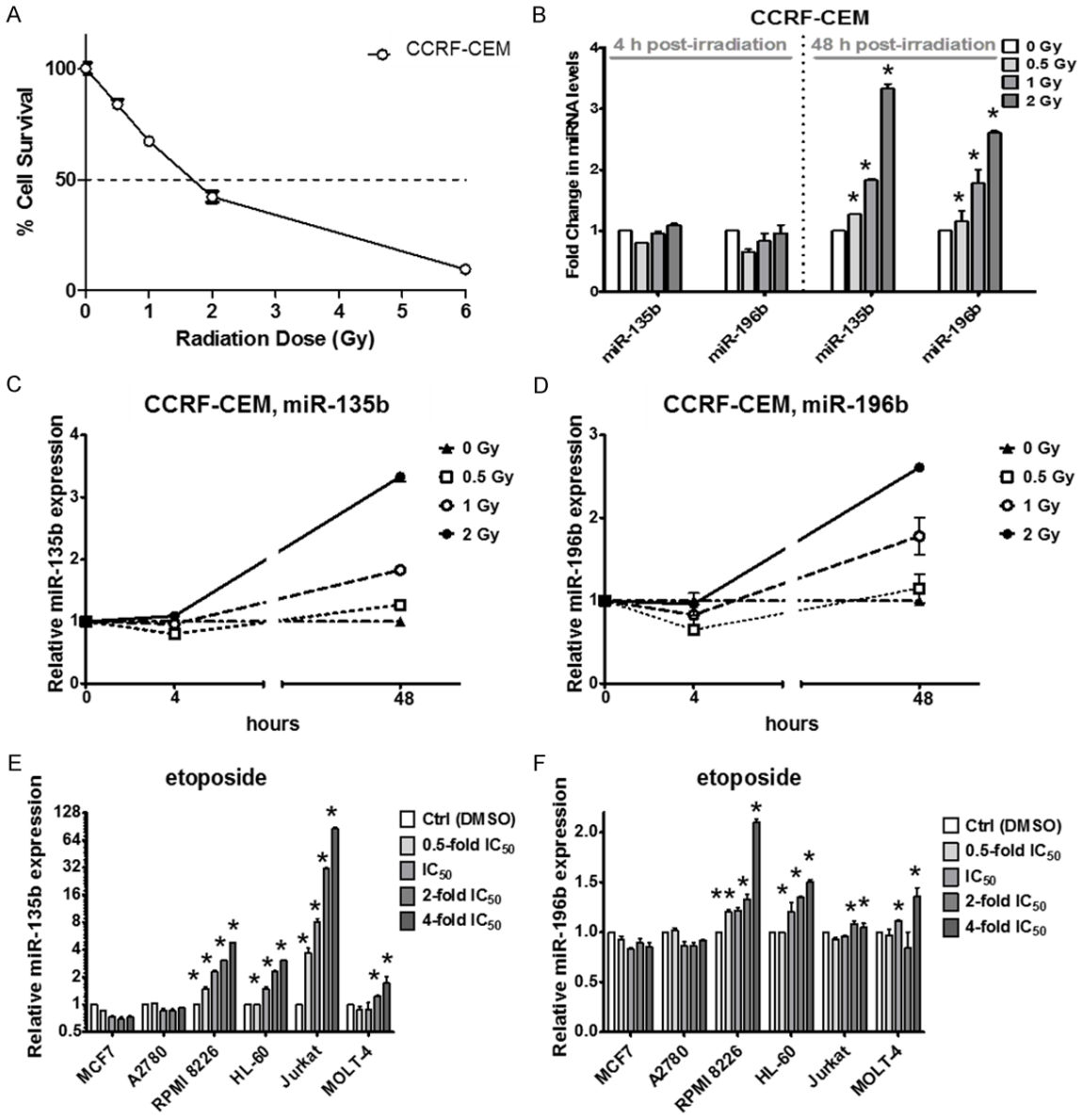

Figure 4. Upregulation of miR-135b and miR-196b following ionizing radiation in CCRF-CEM cells and in response to etoposide in other leukemic cell lines. (A) CCRF-CEM cells were irradiated to doses ranging from 0 Gy to 6 Gy and ID_{50} for CCRF-CEM cells was determined by the dose-response effect of ionizing radiation. (B) Samples were collected 4 h and 48 h after irradiation and evaluated by RT-PCR for expression of miR-135b and miR-196b. A dose-dependent increase in expression from 0.5 Gy to 2 Gy was observed at 48 h post-irradiation in CCRF-CEM cells. In addition, we performed RT-PCR using cells collected at several time points after radiation exposure ranging from 4 h to 48 h. An increase in (C) miR-135b and (D) miR-196b expression was observed at 48 h after irradiation. (E) miR-135b and (F) miR-196b expression were determined in solid tumors-derived MCF7 (breast cancer) and A2780 (ovarian cancer) cell lines, leukemic cell lines: RPMI 8226 (myeloma), HL-60 (acute promyelocytic leukemia), Jurkat (acute T cell leukemia) and MOLT-4 (acute T cell leukemia) cell lines, in response to etoposide for 48 h at the indicated doses. Expression of miR-135b and miR-196b increased after treatment of etoposide for 48 h in leukemia cell lines: RPMI 8226, HL-60, Jurkat, and MOLT-4. No significant changes in expression of miR-135b and miR-196b were observed in cells derived from solid tumors (MCF7 and A2780). Total RNA was collected after 48 h drug incubation, respectively. Values are mean ± SE (n = 3). *, $p < 0.05$.

196b after short-term chemotherapeutic drug exposure was limited to the CCRF-CEM cell line or whether it was a general phenomenon. To test this, we challenged both MCF7 (breast

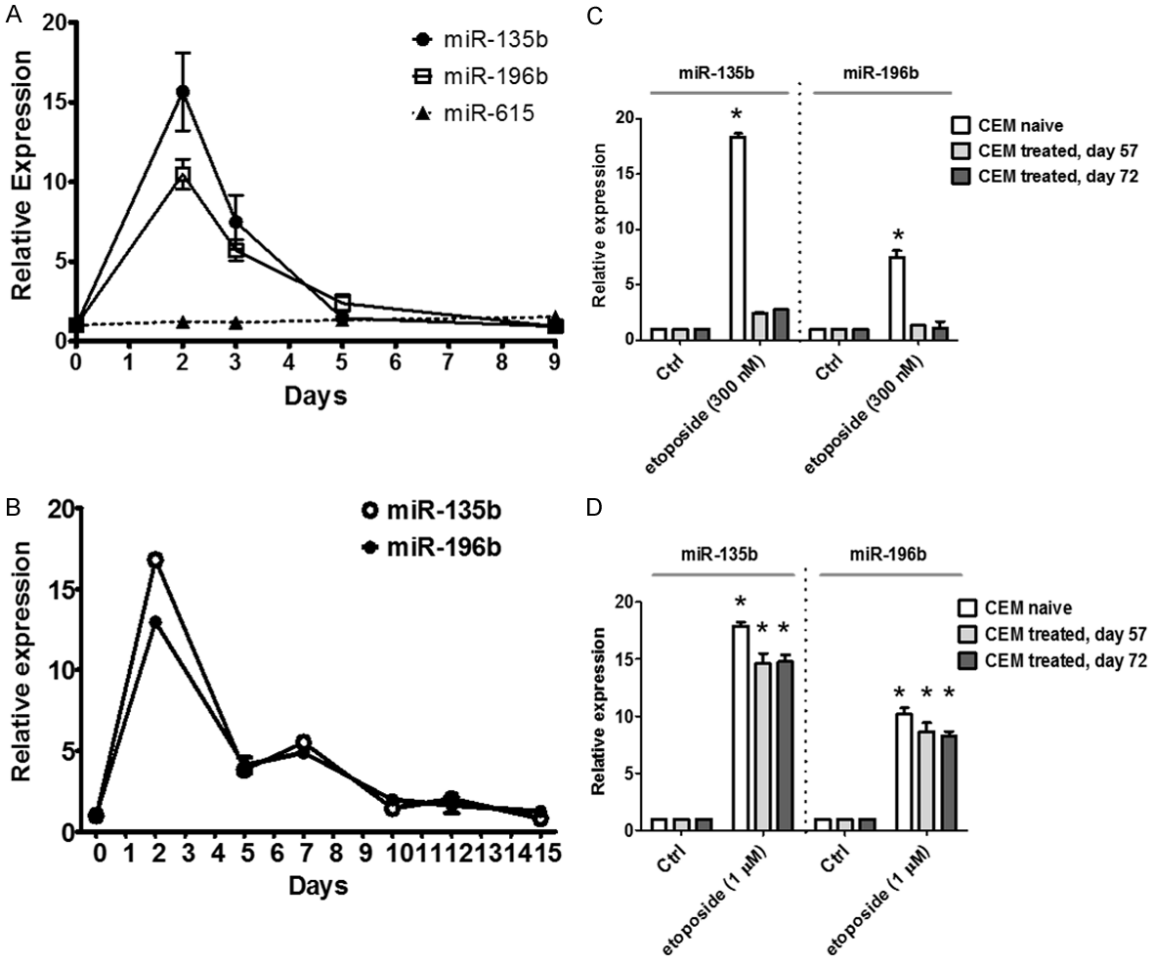

Figure 5. Elevated expression of miR-135b and miR-196b after short-term chemotherapeutic drug exposure. A. Exposure of CCRF-CEM cells to etoposide for 48 h led to upregulation of miR-135b and miR-196b expression which gradually decreased after withdrawal of etoposide, whereas miR-615 served as a negative control. B. Subsequent etoposide (IC_{50}, 300 nM) rechallenges (2nd and 3rd challenge, days 5 and 10) led to progressively attenuated expression of miR-135b and miR-196b compared to their expression after the first challenge using the same etoposide concentration. C. Treatment of these previously-exposed cells again with 300 nM etoposide resulted in attenuation of miR-135b and miR-196b induction response. D. Treatment of these previously-exposed cells again with 1 μM etoposide led to induction of miR-135b and miR-196b expression. Naïve CCRF-CEM cells were used as positive control. Values are mean ± SE (n = 3). *, $p < 0.05$.

cancer) and A2780 (ovarian cancer) cell lines with etoposide at either 0.5-, 1-, 2-, or 4-fold IC_{50} concentrations for 48 h and found no significant changes in expression of miR-135b (**Figure 4E**) and miR-196b (**Figure 4F**). However, we did observe increases in miR-135b and miR-196b expression when we exposed RPMI 8226 (myeloma), HL-60 (acute promyelocytic leukemia), Jurkat (acute T cell leukemia), and MOLT-4 (acute T cell leukemia) cell lines to the indicated doses of etoposide after 48 h incubation, suggesting that induction of expression of miR-135b and miR-196b is not limited to the CCRF-CEM cell line, but may be histiotype-specific. Together, our results suggest that these microRNAs might play a role in the responses of leukemic cells to genotoxic agents; indeed, we suggest that upregulation of miR-135b and miR-196b may be markers of acute DNA damage in leukemic cells.

Elevated expressions of miR-135b and miR-196b after short-term chemotherapeutic drug exposure are associated with the acquisition of transient drug resistance

Given that both miR-135b and miR-196b were upregulated in leukemia cells after short-term chemotherapeutic drug exposure, we asked

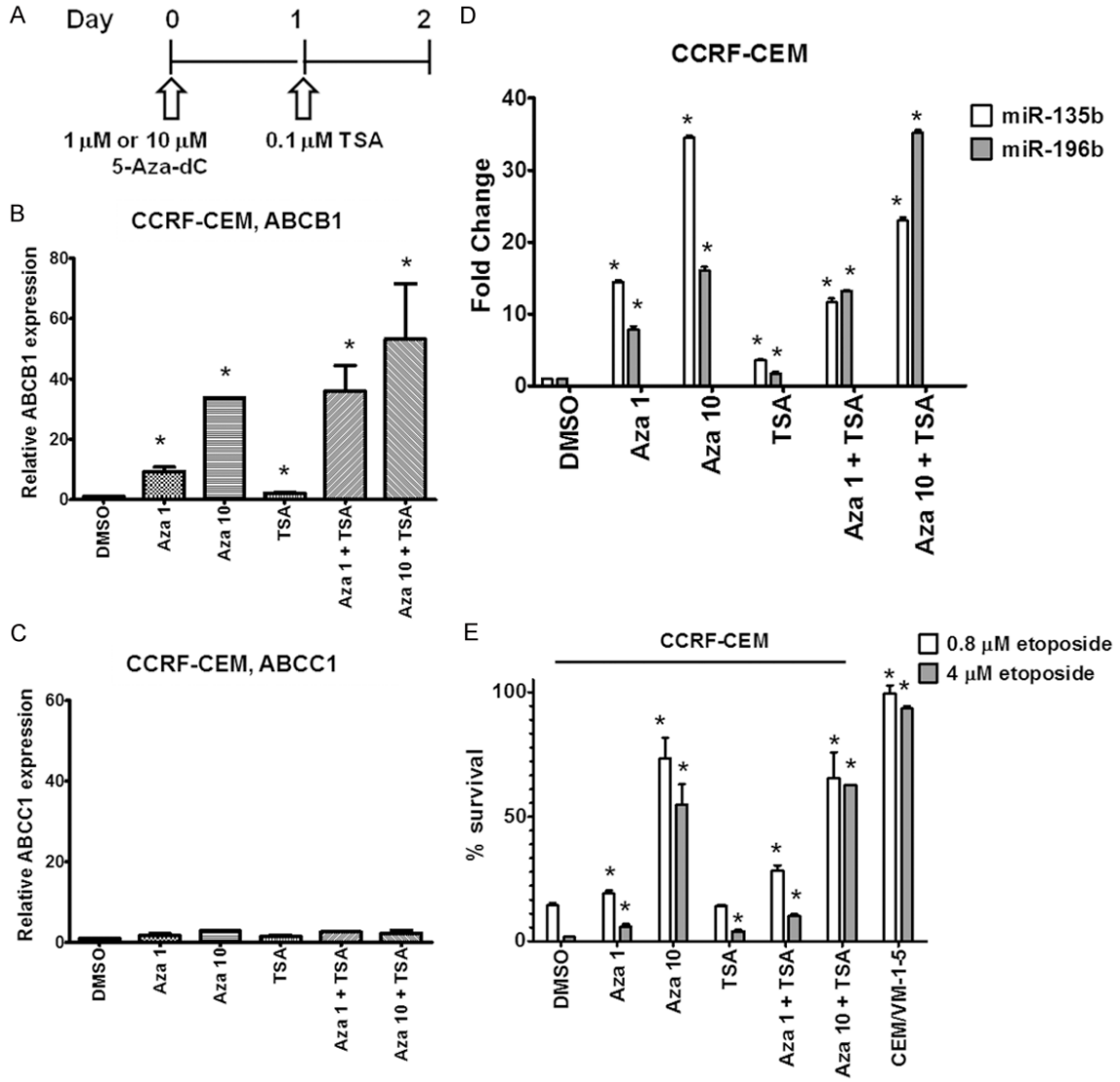

Figure 6. Expression of ABCB1, miR-135b, and miR-196 is regulated epigenetically in CCRF-CEM cells. (A) Scheme of the experimental design. CCRF-CEM cells were treated with the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dC) at 1 μM (Aza 1) or 10 μM (Aza 10) alone, the histone deacetylase inhibitor Trichostatin A (TSA) (0.1 μM) alone, or combinations of both agents. Expression of (B) ABCB1, (C) ABCC1, (D) miR-135b and miR-196b were measured relative to GAPDH (ABCB1 and ABCC1) or RNU48 snoRNA (miR-135b and miR-196b) and normalized to vehicle control (DMSO). Treatment of CCRF-CEM cells with 5-Aza-dC, TSA or both (5-Aza-dC+TSA) led to enhanced expression of ABCB1, miR-135b, and miR-196b (up to 35-fold) compared to untreated cells. (E) CCRF-CEM cells following 5-Aza-dC, TSA or both (5-Aza-dC+TSA) resulted in increase in number of surviving cells. Cell viability assay was used to determine the resistance of CCRF-CEM cells to etoposide at indicated concentrations. Untreated CEM/VM-1-5 cells served as positive control. Values are mean ± SE (n = 3). *, $p < 0.05$.

whether the increase in miR-135b and miR-196b seen in response to chemotherapeutic challenge is associated with the development of etoposide resistance by leukemic cells. To this end, we conducted washout and re-challenge experiments to investigate the kinetics of miR-135b and miR-196b expression. As shown in **Figure 5A**, we found that the elevated expression of miR-135b and miR-196b in CCRF-CEM cells in response to 48 h etoposide exposure correlates well with the increased expression of ABCB1 shown in **Figure 2A**. In addition, this elevated expression of miR-135b and miR-196b, progressively decreased with increased time of incubation of the cells in drug-free medium. Of interest, consistent with

Figure 2B, we also found that subsequent etoposide (IC_{50}, 300 nM) re-challenges (2^{nd} and 3^{rd} challenge, days 5 and 10) led to progressively attenuated expression of the miR-135b and miR-196b genes, compared to their expression after the first challenge using the same etoposide concentration (**Figure 5B**). These results suggest that these two miRNAs are involved, along with ABCB1, in the initial response of cancer cells to chemotherapeutic challenges by DNA damaging agents.

We next asked whether expressions of these two microRNAs are associated with transient drug resistance as we found in **Figure 2D**, **2E**. To this end, we measured expressions of miR-135b and miR-196b in CCRF-CEM cells exposed to repeated drug challenge after either 15 or 20 passages in drug-free medium. Treatment of these previously-exposed cells again with 300 nM etoposide resulted in unexpected attenuation of miR-135b and miR-196b expression (**Figure 5C**). When we treated these previously-exposed cells with 1 μM etoposide and measured expressions of miR-135b and miR-196b, we found that the expressions of miR-135b and miR-196b were induced as expected (**Figure 5D**). These results suggest that miR-135b and miR-196b may be involved in the initial events of CCRF-CEM cells in response to anticancer agents.

Expressions of ABCB1, miR-135b, and miR-196b are regulated epigenetically in leukemic cells

It has been reported that genes encoding miRNAs undergo the same regulatory mechanisms as conventional protein-coding genes, including epigenetic regulation [22]. To develop a mechanistic understanding of why these microRNAs are increased in expression after short-term exposure to DNA damaging agents, we examined their genomic locations and found that the upstream regions of the miR-135b and miR-196b loci are embedded in CpG islands, suggesting that DNA demethylation may activate the expression of these miRNAs. To determine whether these microRNAs are altered in response to epigenetic modifiers, we treated CCRF-CEM cells with 1 μM or 10 μM of the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dC) and/or 0.1 μM of the histone deacetylase inhibitor Trichostatin A (TSA) for 48 h as indicated (**Figure 6A**). As shown in **Figure 6B-D**, we found that treatment of cells with these agents either alone or combination led to enhanced expression of ABCB1, miR-135b and miR-196b (up to 35-fold), but not ABCC1, compared to untreated cells. These results suggest that epigenetic mechanisms could be involved in the expression of ABCB1, miR-135b and miR-196b. We then asked whether these cells treated with 5-Aza-dC and TSA have altered sensitivity to etoposide, as assessed by MTS assay. As seen in **Figure 6E**, we found that the treatment of CCRF-CEM cells with epigenetic modifiers decreases their sensitivity to etoposide by up to 5- or 36-fold, depending on the concentrations of etoposide used, thus correlating positively with increased percentage of survival (drug resistance) in the presence of drug. In addition, this apparent "acquired drug resistance" appears to be due more to 5-Aza-dC (demethylation) than to TSA (histone deacetylase). These results suggest that the induced expressions of ABCB1, miR-135b and miR-196b in CCRF-CEM cells along with acquired drug resistance can be regulated epigenetically.

Ectopic expression of miR-135b and miR-196b in CCRF-CEM cells confers modest resistance to teniposide and changes morphological features

To investigate whether miR-135b and miR-196b have direct or indirect functions in drug resistance, we ectopically expressed miR-135b and miR-196b in CCRF-CEM cells using a lentiviral system. CCRF-CEM cells transduced with miR-135b or miR-196b singly or in combination exhibited modest (~2-3 fold) increases in resistance to teniposide, a close analog of etoposide; we used teniposide in this experiment because it was the drug used originally to select CEM/VM-1-5 cells [26] (**Figure 7A**). However, it was not surprising that ectopic expression of miR-135b or miR-196b in CCRF-CEM cells did not confer drug resistance to the same extent as the selected CEM/VM-1-5 cells, which were exposed to teniposide for many passages and have many changes, including mutant topoisomerase II α, all leading to their 400-fold resistance to teniposide, and 129-fold cross-resistance to etoposide [35]. In a complementary experiment, knockdown of miR-135b in CEM/VM-1-5 cells with LNA-antagomiR-135b

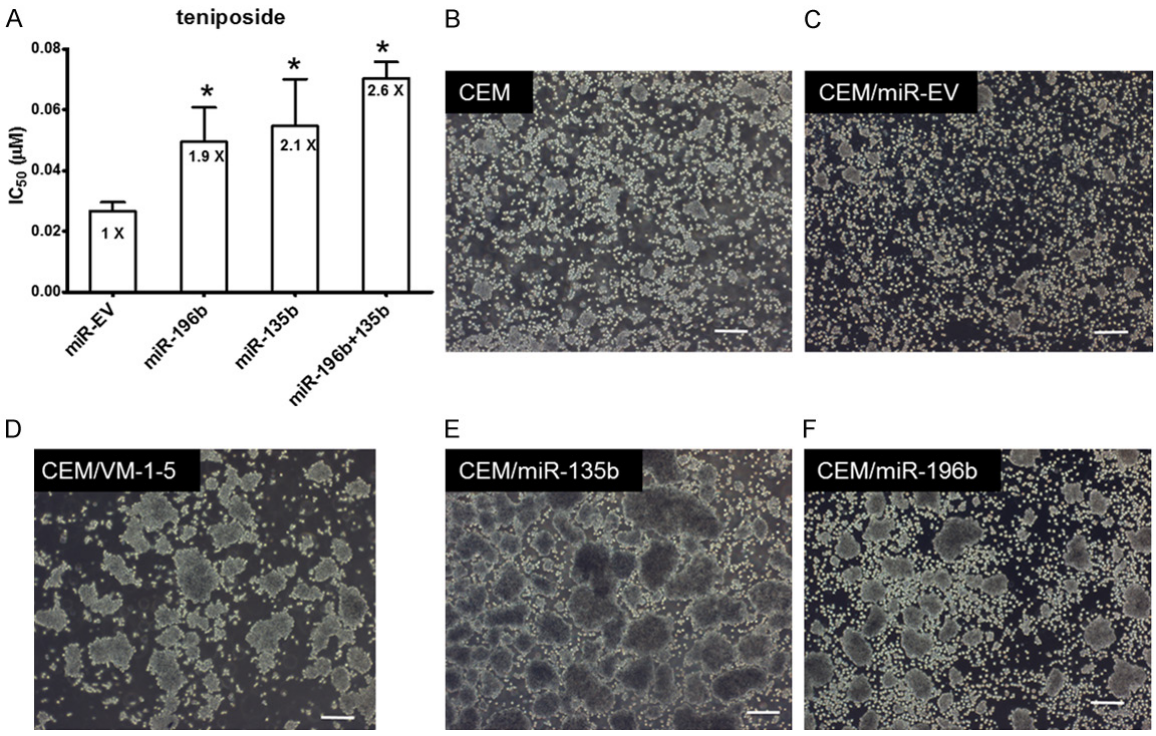

Figure 7. Ectopic expression of miR-135b and miR-196b in CCRF-CEM cells confers modest resistance to teniposide and changes morphological features. Forced expression of miR-135b or miR-196b in CCRF-CEM cells was performed with the use of lentiviral transduction. (A) IC_{50} values of CCRF-CEM cells transduced with miR-135b or -miR-196b, individually or in combination, to teniposide were assessed using modified MTT assay and calculated by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). After infection, the CCRF-CEM cells (B) underwent morphological changes. Infected (E) CEM/miR-135b cells and (F) CEM/miR-196b cells became clustered and looked like (D) CEM/VM-1-5 cells, while no significant changes were seen in (C) CEM/miR-EV cells transduced with control virus. EV: empty vector control. Scale bar: 200 μm. Values are mean ± SE (n = 3). *, $p < 0.05$.

did not sensitize these cells to etoposide (data not shown). Therefore, we speculate that miR-135b and miR-196b may play a role in the development of drug resistance, but not in the maintenance of this state. Of note, after ectopic expression of miR-135b and miR-196b, the CCRF-CEM cells underwent morphological changes. Infected-cells became clustered and looked like their resistant counterpart CEM/VM-1-5 cells (**Figure 7B-F**), suggesting that these microRNAs are associated with some likely alterations in cell membrane properties associated with MDR in these cells.

Predicted targets of miR-135b and miR-196b assessed by miRNA-proteomics

To identify potential proteins involved in this phenotypic change, we carried out a bioinformatics study to attempt to identify computationally predicted targets of miR-135b and miR-196b. Computational prediction of targets against miRNAs typically leads to hundreds of predicted targets and is widely held to be susceptible to false positive prediction [36]. Thus, we used consensus prediction of targets, considering only targets commonly predicted through three different prediction programs: miRanda [37], TargetScan [38], and PicTar [39], to generate a list of targets for miR-135b and miR-196b. For miR-135b, miRanda predicts 1113 targets, whereas TargetScan and PicTar predict 510 and 428 targets, respectively, for the same miRNA. For miR-196b, miRanda predicts 1119 targets, whereas TargetScan and PicTar predict 211 and 162 targets, respectively, for the same miRNA. This analysis revealed 33 common predicted targets for miR-135b and 12 for miR-196b respectively with no overlaps using a strict cutoff of $p < 0.01$ for significance. The lists of common predicted targets obtained are provided in **Table 2**.

Enriched annotation terms for predicted targets for miR-135b and miR-196b

The changes in the proteome can provide valuable insights about the state of the cells follow-

Table 2. Common predicted targets of (A) miR-135b and (B) miR-196b by miRanda, TargetScan and PicTar

A.	
Common predicted targets of miR-135b	
AEBP2	AE binding protein 2
ARHGAP6	Rho GTPase activating protein 6
ARHGEF2	Rho/rac guanine nucleotide exchange factor (GEF) 2
ARHGEF4	Rho guanine nucleotide exchange factor (GEF) 4
BZW2	Basic leucine zipper and W2 domains 2
CACNA1D	Calcium channel, voltage-dependent, L type, alpha 1D subunit
CACNA1E	Calcium channel, voltage-dependent, R type, alpha 1E subunit
CALN1	Calneuron 1
CTTNBP2	Cortactin binding protein 2
DPF1	D4, zinc and double PHD fingers family 1
DUSP5	Dual specificity phosphatase 5
FKBP1A	FK506 binding protein 1A, 12 kDa
FRK	Fyn-related kinase
HOXA10	Homeobox A10
INHBA	Inhibin, beta A
MRAS	Muscle RAS oncogene homolog
NR3C2	Nuclear receptor subfamily 3, group C, member 2
ORC5L	Origin recognition complex, subunit 5-like (yeast)
PHOSPHO1	Phosphatase, orphan 1
PTK2	Protein tyrosine kinase 2
SIAH1	Seven in absentia homolog 1 (Drosophila)
SLC24A2	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2
SP3	Sp3 transcription factor
SSR2	Signal sequence receptor, beta (translocon-associated protein beta)
SUV420H2	Suppressor of variegation 4-20 homolog 2 (Drosophila)
TBK1	TANK-binding kinase 1
TMEM9	Transmembrane protein 9
TRPC1	Transient receptor potential cation channel, subfamily C, member 1
USP15	Ubiquitin specific peptidase 15
WAC	WW domain containing adaptor with coiled-coil
ZDHHC6	Zinc finger, DHHC-type containing 6
ZNF143	Zinc finger protein 143
ZNF322A	Zinc finger protein 322A
B.	
Common predicted targets of miR-196b	
ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9
C20orf160	Chromosome 20 open reading frame 160
CDYL	Chromodomain protein, Y-like
COL1A2	Collagen, type I, alpha 2
HAND1	Heart and neural crest derivatives expressed 1
HOXA5	Homeobox A5
HOXB6	Homeobox B6
HOXB7	Homeobox B7
HOXC8	Homeobox C8
MGAT4A	Mannosyl (alpha-1,3-)-glycoprotein beta-1, 4-N-acetylglucosaminyltransferase, isozyme A
NME4	non-metastatic cells 4
SMCR8	Smith-Magenis syndrome chromosome region, candidate 8

Table 3. Classification of miR-135b predicted targets: enriched annotation terms

Terms†	No. of Genes	Gene Symbol	Note: GO criteria
GO: cytoskeleton organization	5	CTTNBP2, ARHGEF2, ARHGAP6, PTK2, MRAS	Biological process (BP)
GO: plasma membrane part	8	ARHGEF4, TRPC1, CTTNBP2, ARHGEF2, PTK2, MRAS, CACNA1E, CACNA1D	Cellular component (CC)
GO: plasma membrane	8	ARHGEF4, TRPC1, CTTNBP2, ARHGEF2, PTK2, MRAS, CACNA1E, CACNA1D	Cellular component (CC)
GO: internal side of plasma membrane	3	ARHGEF4, PTK2, MRAS	Cellular component (CC)
KEGG: Regulation of actin cytoskeleton	3	ARHGEF4, PTK2, MRAS	
SP PIR Keywords: cell membrane	4	ARHGEF4, PTK2, MRAS, CALN1	

†Categories analyzed included Gene Ontology (GO) categories, pathways database [Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways], and functional categories [Swiss-Prot (SP) and Protein Information Resource (PIR) Keywords].

Table 4. Gene expression and phenotype association for miR-135b and miR-196b predicted targets

Populations	Etoposide	Chrom	Gene	*p*-value	Predicted target for
CEU	AUC	1	CACNA1E	8.88E-05	miR-135b
CEU	IC_{50}	1	CACNA1E	1.29E-06	miR-135b
CEU	AUC	1	ARHGEF2	1.92E-03	miR-135b
CEU	IC_{50}	1	ARHGEF2	2.05E-03	miR-135b
CEU	AUC	8	PTK2	1.26E-04	miR-135b
CEU	IC_{50}	8	PTK2	5.48E-03	miR-135b
YRI	AUC	16	SIAH1	2.92E-02	miR-135b
YRI	IC_{50}	16	SIAH1	2.30E-02	miR-135b
YRI	IC_{50}	X	ARHGAP6	3.16E-02	miR-135b
CEU	AUC	X	ARHGAP6	1.39E-02	miR-135b
CEU	IC_{50}	X	ARHGAP6	1.19E-02	miR-135b
YRI	IC_{50}	16	NME4	2.93E-02	miR-196b

CEU: Caucasians from Utah, USA; YRI: Yoruba people from Ibadan, Nigeria; AUC: the area under the cellular survival curve; IC_{50}: the concentration necessary to inhibit 50% of cell growth.

ing short-term anticancer drug exposure and shed light on the overall effect of miR-135b and miR-196b. To understand the cellular roles of these miRNA predicted targets as revealed through proteomics, we performed functional classification of these targets using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (http://david.abcc.ncifcrf.gov/home.jsp) [40]. Of interest, Gene Ontology (GO) [41] classification of predicted targets of miR-135b revealed the occurrence of the GO terms, "plasma membrane part" and "cell membrane" related terms to be involved, whereas GO classification of miR-196b predicted targets enriched in "pattern specification process" and "embryonic morphogenesis". GO terms linked to membrane are listed in **Table 3**, indicating these genes may play a role in changes in cell morphology shown in **Figure 7E**, **7F**. However, to be secure in this conclusion, we will need to perform functional analysis of the proteins predicted by these genes.

Gene expression of predicted targets for miR-135b and miR-196b and phenotype association

Given that miR-135b and miR-196b were upregulated following short-term exposure to DNA-damaging agents, we then searched Pharmacogenomics and Cell database (PACdb) (http://www.pacdb.org/) [42], which integrates gene expression and pharmacological data obtained via lymphoblastoid cell lines, to investigate if any of the targets commonly predicted through miRanda, TargetScan, and PicTar for miR-135b and miR-196b are associated with drug response. As shown in **Table 4**, we found that among 33 common predicted targets for miR-135b, calcium channel, voltage-dependent, R type, alpha 1E subunit (CACNA1E), Rho/rac guanine nucleotide exchange factor 2 (ARHGEF2), protein tyrosine kinase 2 (PTK2), seven in absentia homolog 1 (SIAH1), rho GTPase activating protein 6 (ARHGAP6) were associated with either the area under the cellular survival curve (AUC) or the concentration necessary to inhibit 50% of cell growth (IC_{50}) of etoposide, the DNA-damaging drug used in our study, and for Caucasians from Utah, USA (CEU) or Yoruba people from Ibadan, Nigeria (YRI) using a default cutoff of $p < 0.05$. We also found that non-metastatic cells 4 (NME4) out of 12 common predicted targets for miR-196b was associated with IC_{50} of etoposide for YRI. These analyses suggest that CACNA1E, ARHGEF2, PTK2, SIAH1, ARHGAP6, and NME4 may be involved in the initial events in the development of drug resistance following the upregulation of miR-135b and -196b in response to chemotherapeutic challenge, and provide us with molecular leads as we attempt to understand these early events in the development of resistance to DNA damaging agents.

Discussion

The acquisition of resistance to chemotherapy is a major clinical obstacle in the treatment of cancer, greatly increasing patient morbidity and mortality [13]. Tumors are often sensitive to chemotherapy upon initial treatment, but repeated treatments can select for those cells that are able to survive initial therapy and have acquired cellular mechanisms to enhance their resistance to subsequent chemotherapy treatment [43]. Many cellular mechanisms of drug resistance have been identified [44]. In our present study, we have illustrated the feasibility of using unbiased miRNA profiling to assist in better understanding mechanisms of MDR, and in doing so, we demonstrated that certain

miRNAs might play a significant role in acute cellular responses to genotoxic agents; upregulation of miR-135b and miR-196b appear to be markers of DNA damage and early drug resistance in leukemia cells. Cells with increased expression of ABCB1, miR-135b, and miR-196b may be able to repair the damages and ultimately survive, while the cells with low level of expression of these genes would die following the treatment. While other miRNAs have been associated with the acquisition of multidrug resistance and expression of ABC transporters [45, 46], and other miRNAs have been associated with the DNA damage response [47], our finding of the association of miRNAs associated with DNA damage-associated drug resistance in leukemic cells appears to be novel. The expanding knowledge of the molecular pathogenesis of anticancer drug resistance is providing new targets for treatment that might also be used as new markers to select patients for better clinical management. In particular, there are a growing number of studies on miRNAs demonstrating that they have a pivotal role in the prediction of resistance for adjuvant chemotherapy [18]. Our data suggest that miR-135b and miR-196b may serve as potential markers predicting chemotherapeutic response and targets for preventing chemotherapeutic resistance in leukemia cells.

We have validated at least five miRNAs differentially expressed in the multidrug-resistant cell line CEM/VM-1-5 compared to drug-sensitive CCRF-CEM human T-cell leukemia cell line from which it was derived. In light of the altered miRNA expression studied extensively in human cancers [48], miRNA expression appears to hold great promise in tumor diagnosis and treatment [49]. Studies to interfere with miRNA function *in vivo* provide novel therapeutic opportunities for cancer treatments [50]. We found herein that transient expression of both miR-135b and miR-196b, which we found to be constitutively upregulated in drug resistant leukemic cells, is also associated with transient anticancer drug resistance. Our results illustrate the involvement of miRNAs in the likely development of drug resistance in a human T-cell leukemia cell line and suggest that miR-135b and miR-196b could serve as potential markers of MDR.

Of interest, we note that expression of these two miRNA species was increased following short-term exposure to genotoxic drugs, including etoposide, doxorubicin and topotecan, but not in cells treated with vinblastine or paclitaxel, suggesting that the upregulation of these two microRNAs might be a consequence of DNA damage. The magnitude of the change in their expression correlated both with drug concentrations and time. To further confirm that upregulation of these two miRNAs is a consequence of DNA damage, we examined their expression following ionizing radiation. As expected, the elevation of miR-135b and miR-196b correlated positively with the time after radiation exposure and radiation dosage, in accordance with what we observed in cells treated with etoposide, a chemotherapeutic agent that generates DNA double strand breaks [51]. Together, our results further confirm that miRNAs might play a significant role in cellular response to genotoxic agents and the DNA damage response [47], and suggest that upregulation of miR-135b and miR-196b may be a marker of acute DNA damage in leukemia cells. Accordingly, we believe that we have uncovered a novel role for these miRNAs as responders to genotoxic stress, with consequent involvement in the development of anticancer drug resistance.

In this study, we used three prediction programs (miRanda, TargetScan, and PicTar) to generate a list of common targets for miR-135b and miR-196b, respectively, and carried out a bioinformatics study to enrich annotation terms for these targets and to investigate the association of these genes and the cellular phenotype (etoposide response). It has been shown that several Homeobox (*Hox*) genes, including HoxA7, HoxB8, HoxC8, and HoxD8 appear to be conserved targets of miR-196b [52, 53]. During vertebrate development, miR-196b-directed cleavage of HoxB8 was detected in mouse embryos [54]; moreover, miR-196b has been shown to be involved in vertebrate development [53], and is also known as one of the hematopoietic miRNAs that modulate hematopoietic lineage differentiation [55]. Co-regulation of protein-encoding and/or miR-encoding genes may have important regulatory consequences in cell physiology by generating feedback loops that avoid uncontrolled expression of protein-coding genes [56]. Since miR-196b is encoded on the HoxA cluster, one study suggested that both miR-196b and HoxA-genes were co-activated in acute lymphoblastic leuke-

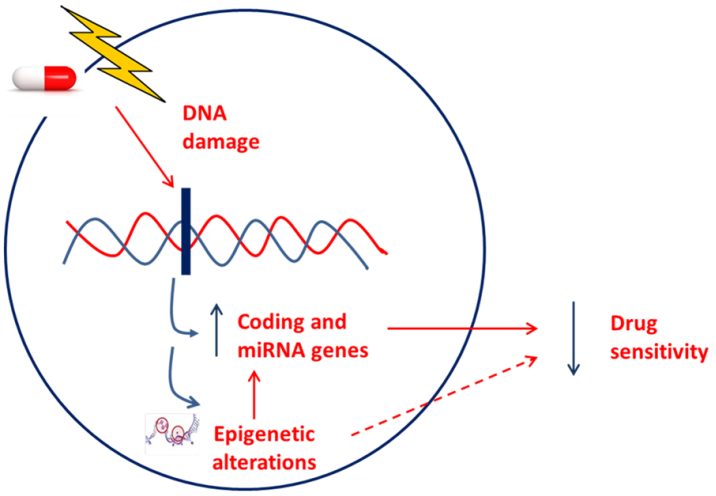

Figure 8. Model summarizing data on transient drug resistance. Transient exposure to DNA-damaging agents, including etoposide, can lead to transient decreased drug sensitivity, or drug resistance, in leukemic cells. This drug resistance is associated with transient expression of a coding gene, ABCB1, as well as non-coding microRNA genes, miR-135b and miR-196b. Further, these genes may be regulated, in part, by epigenetic mechanisms.

mia (ALL) [57]. It has been reported that treatment of leukemia by ATRA and As_2O_3 may be associated with the regulation of HoxA9 expression in that these treatments led to increased HoxA9 mRNA expression, followed by a decrease [58]. Therefore, whether there is a causal relationship between HoxA-genes and transient anticancer drug resistance is worth further investigation.

As miR-196b is located on chromosome 7p15.2, between the loci of HoxA9 and HoxA10, their co-expression suggests that they might be co-regulated. However, it has been demonstrated that miR-196b directly targets both HoxA9/MEIS1 oncogenes in MLL-rearranged leukemia [59]. One possible explanation for this apparent discrepancy is the known diverse nature of miRNA target genes. The net effect of changes in the expression of a miRNA appears to be the sum of its effects on all of its targets in a cell type-specific and phenotype-specific manner [18]; this would support the idea that miRNAs mediate regulation of a dynamic balance among target genes through a context-dependent manner.

The mechanism underlying the differential expression of miR-135b and miR-196b in association with and possibly conferring anticancer drug resistance in leukemia cells remains to be determined. One possible explanation is that these genes reside in genetically unstable regions. Indeed, miR-196b is in one such region (chr7p15) that is prone to translocations in MOLT-4, a T-cell ALL-derived cell line [60]. Moreover, it has been shown that miR-196b is a downstream target of Mixed Lineage Leukemia (MLL) [61]. Hox genes represent one set of transcriptional targets that warrant assessment in leukemias with MLL translocation [62]. Thus, to address the causes of the defects leading to the imbalance of miRNAs in drug-resistant cancer cells, further investigation of the expression of the various MLL fusions is warranted.

miR-135b is located in the first intron of the LEM domain containing 1 (LEMD1) gene that is highly expressed in colorectal cancer (CRC) [63], and has been implicated in CRC progression through the PTEN/PI3K pathway [64]. Moreover, it has been shown that Jurkat cells overexpressing miR-135b were more resistant to cytosine β-D-arabinofuranoside, suggesting that miR-135b expression may confer chemoresistance [65]. This is consistent with our finding that miR-135b is constitutively upregulated in our drug-resistant cells, although we do not know about their sensitivity to this nucleoside analog [26]. Additionally, our results implicate miR-135b in the development of drug resistance, consistent with the finding that miR-135b is involved in the radioresistance in human glioblastoma multiforme cells [66]. Of interest, it has been reported that chromosomal regions containing differentially expressed miRNAs also show alteration in DNA methylation status in colon cancer [67]. Moreover, the reversibility of resistance of T-cell acute lymphoblastic leukemia cells to γ-secretase inhibitors indicates an epigenetic mechanism [68]. Therefore, the cause of the differential expression of miR-135b could be explained by epigenetic alterations, in accordance with our findings shown in **Figure 6D**.

In conclusion, our results suggest the following model (**Figure 8**): we have shown in this study that transient exposure to DNA-damaging agents, including etoposide, can lead to tran-

sient decreased drug sensitivity, or drug resistance, in leukemic cells. This drug resistance is associated with transient expression of a coding gene, ABCB1, as well as non-coding microRNA genes, miR-135b and miR-196b. Further, these genes may be regulated, in part, by epigenetic mechanisms. Finally, our bioinformatics analyses point to novel directions and genes to examine as these studies progress.

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Disclosure of conflict of interest

None.

Authors' contribution

Participated in research design: Ho, He, and Beck. Conducted experiments: Ho and He. Contributed new reagents or analytic tools: Mo. Performed data analysis: Ho and He. Wrote or contributed to the writing of the manuscript: Ho and Beck.

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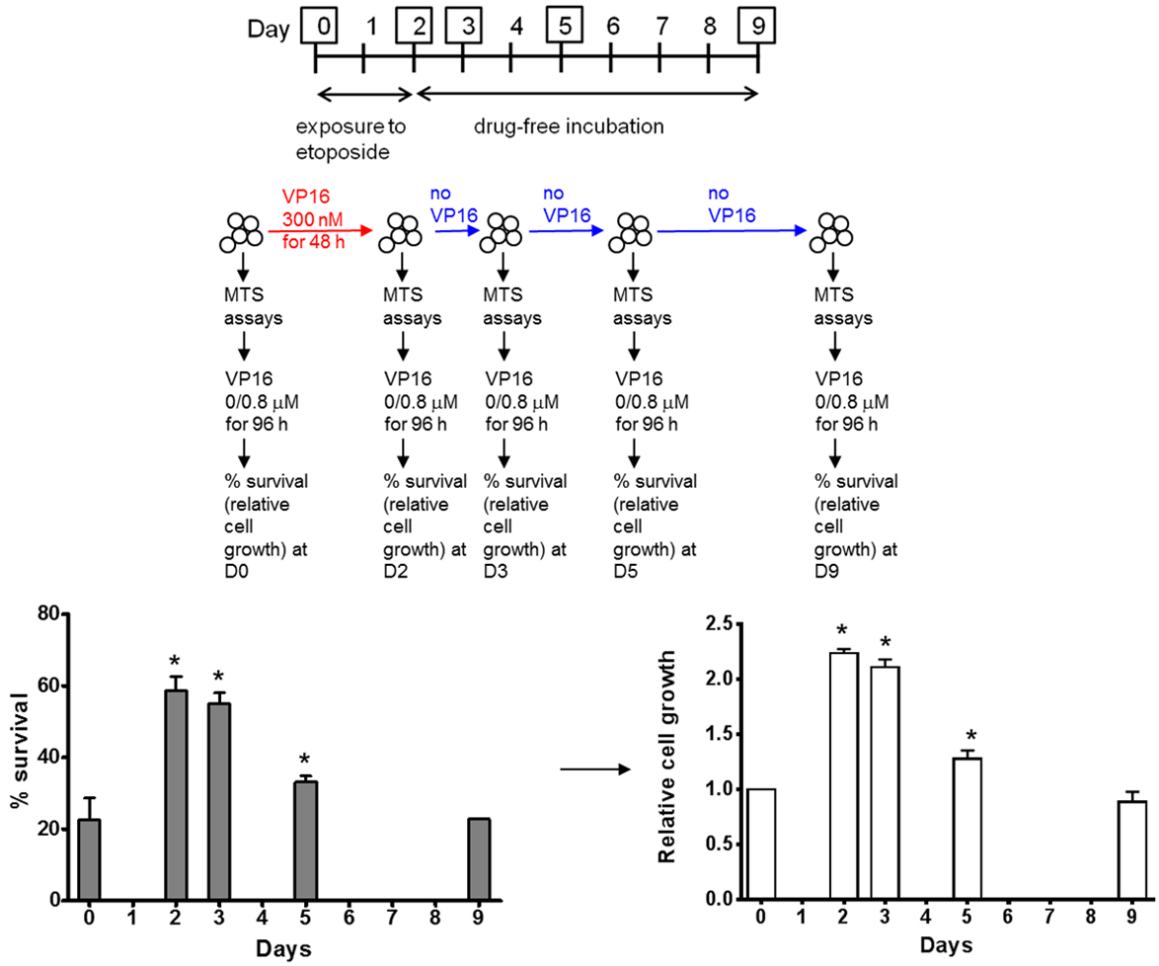

Figure S1. Schematic of experimental protocol for **Figure 1B**. At indicated time points, we performed MTS assays to determine the relative etoposide sensitivity or resistance of CCRF-CEM cells. At each stage, including that for Day zero (D0), we incubated CCRF-CEM cells with 0.8 μM etoposide or vehicle (DMSO) for 96 h, and then converted % survival (lower left histogram) to relative cell growth (lower right histogram) (VP16 300 nM 48-h treated cells/vehicle treated cells). The lower right histogram is reproduced in **Figure 1B**.