Correcting miR-15a/16 genetic defect in New Zealand Black mouse model of CLL enhances drug sensitivity

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Abstract

Alterations in the human 13q14 genomic region containing microRNAs mir-15a and mir-16-1 are present in most human chronic lymphocytic leukemia (CLL). We have previously found the development of CLL in the New Zealand Black murine model to be associated with a point mutation in the primary *mir-15a/16-1* region, which correlated with a decrease in mature miR-16 and miR-15a levels. In this study, addition of exogenous miR-15a and miR-16 led to an accumulation of cells in G₁ in non-New Zealand Black B cell and New Zealand Black-derived malignant B-1 cell lines. However, the New Zealand Black line had significantly greater G₁ accumulation, suggesting a restoration of cell cycle control upon exogenous miR-15a/16 addition. Our experiments showed a reduction in protein levels of cyclin D1, a miR-15a/16 target and cell cycle regulator of G₁/S transition, in the New Zealand Black cell line following miR-15a/16 addition. These microRNAs were shown to directly target the cyclin D1 3' untranslated region using a green fluorescent protein lentiviral expression system. miR-16 was also shown to augment apoptosis induction by nutlin, a mouse double minute 2 (MDM2) antagonist, and genistein, a tyrosine kinase inhibitor, when added to a B-1 cell line derived from multiple in vivo pas-

Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

sages of malignant B-1 cells from New Zealand Black mice with CLL. miR-16 synergized with nutlin and genistein to induce apoptosis. Our data support a role for the *mir-15a/ 16-1* cluster in cell cycle regulation and suggest that these mature microRNAs in both the New Zealand Black model and human CLL may be targets for therapeutic efficacy in this disease. [Mol Cancer Ther 2009;8(9):2684–92]

Introduction

MicroRNAs are small, evolutionarily conserved, noncoding single-stranded RNAs that regulate gene expression by binding with a RNA-induced silencing complex (RISC)-like complex to the 3' untranslated region (UTR) of target mRNA (1, 2). Loss or amplification of microRNAs are reported in various cancers, affecting cell cycle progression or survival mechanisms (3) by acting as either tumor suppressors or oncogenes (4).

Chronic lymphocytic leukemia (CLL), a malignancy of the CD5⁺ B cell, is the most common leukemia to affect adults in the Western world (5). More than 50% of CLL cases exhibit a deletion within the 13q14 chromosomal region (6) containing the *DLEU2* gene (7, 8). *DLEU2* contains microRNAs *mir-15a* and *mir-16-1*, which are also deleted or down-regulated in a subpopulation of patients with B-cell CLL (9, 10). The New Zealand Black mouse, a *de novo* model for autoimmunity (11) and CLL (12, 13), exhibits a T \rightarrow A point mutation six bases downstream from *pre–mir-16-1* on chromosome 14 (14), similar to the C \rightarrow T point mutation seen in human CLL (15), which may affect structural stability of the stem loop and proper processing, resulting in decreased miR-15a and miR-16 expression.

Similar to CLL, the disease in New Zealand Black mice is an age-associated malignant expansion of poly-reactive CD5⁺, B-1 clones (13, 16). Most B-1 clones are IgM⁺, B220 (CD45R)^{dull}, and CD5^{dull}, and often possess chromosomal abnormalities (17). The germline genetic alterations in the *mir-16-1* locus in New Zealand Black mice are correlated with a decrease in mature miR-16 expression in lymphoid tissues (14). The New Zealand Black also displays mild autoimmunity associated with B-cell hyperactivity, resulting in autoimmune hemolytic anemia and antinuclear antibodies (13), similar to autoimmunity seen in some CLL patients, resulting from the production of autoantibodies (18).

CLL was classically defined as a progressive accumulation of resting B cells possessing a defect in the apoptotic pathway (19). Although leukemic cells circulating in the peripheral blood have been shown to be arrested in G_0/G_1 (20), CLL is now thought to be more dynamic, with a detectable level of cell division occurring at all times (21). In lymphoid tissue, leukemic cells appear as aggregates termed "pseudofollicles" or "growth centers," in which neighboring CD4⁺ CD40L⁺ T cells induce their proliferation (20).

Received 2/12/09; revised 5/19/09; accepted 6/11/09; published OnlineFirst 9/1/09.

Grant support: This research was funded in part by the New Jersey Commission on Cancer Research predoctoral fellowship awarded (E. Salerno).

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MicroRNAs have been found to have an impact on cell cycle progression (22–25). MicroRNAs, such as miR-106b, miR-221, and miR-222, have been found to promote cell cycle progression in various human solid tumors (22, 23), and miR-16 and let-7b have been reported to contribute to G_1 accumulation in human colon carcinoma cell lines and melanoma cells, respectively (24, 26).

In this study, we sought to define a role for miR-16 in the malignant B-1 cell growth and persistent nature seen in the New Zealand Black model of CLL by examining the effects of exogenous miR-16 on our New Zealand Black-derived malignant B-1 cell line (LNC) that mimics the late stages of CLL (27). We were interested in the effects of miR-16 on its targeted cell cycle components, particularly cyclin D1. Although overexpression of cyclin D1 among leukemias and lymphomas is a hallmark of mantle cell lymphoma, resulting from translocation t(11;14)(q13;q32) (28, 29), increased expression of cyclin D1 has been found in some patients with CLL (30), resulting in poor prognosis (31). Recent studies in carcinomas and prostate cancer have also supported the notion that cyclin D1 is indeed a target of and regulated in part by miR-16 (32, 33). We were also interested in the role miR-16 may play in augmenting apoptotic effects of chemotherapy. We used nutlin, a smallmolecule MDM2 antagonist and p53 activator (34), and genistein, a soy isoflavone tyrosine kinase inhibitor known to down-regulate cyclin D1 (35), as agents to induce apoptosis and block proliferation in our robust New Zealand Black-derived malignant B-1 cell line. The results of our study suggest that aberrant expression of miR-16 plays a role in deregulated cell cycle transit and drug sensitivity, and upon restoration of miR-16 levels to malignant B-1 cells, uncontrolled proliferation can be impeded and selective killing of malignant B-1 cells can be enhanced.

Materials and Methods

Sources of Cells

Mice, New Zealand Black/BlNJ and C57Bl/6, were obtained from Jackson Laboratories. The New Zealand Black–derived malignant B-1 cell line, LNC, was derived from a year-old New Zealand Black, as previously described (27). The BALB/c derived B-cell line, A20, was obtained from American Type Culture Collection. The NFS-1.0 B-cell line was derived from the NFS/N strain (36) and a kind gift from Dr. Steven Bauer, Center for Biologics Evaluation and Research, Food and Drug Administration. A20 and NFS-1.0 were used as non–New Zealand Black B-cell lines (both having normal levels of miR-15a and miR-16), and C57Bl/6 mice were used as non–New Zealand Black mice, having normal levels of miR-15a and miR-16.

Cell Sorting of Spleen Cells

Spleens were removed from 9- to 14-mo-old C57B1/6 (control non–New Zealand Black strain) and New Zealand Black mice; made into single-cell suspensions that were four-color stained with antibodies directed against IgM fluorescein isothiocyanate, B220 PerCP-Cy5.5, CD4 phycoerythrin, and CD25 allophycocyanin (Invitrogen); and

sorted into four distinct populations on a FACSAria (Becton Dickinson). The B cells were gated on CD4⁻ and sorted into two populations (B-2 cells, CD4⁻/IgM⁺/B220^{bright}; B-1 cells, CD4⁻/IgM⁺/B220^{dull}). T cells were sorted into two populations (IgM⁻/CD4⁺/CD25⁻ and Tregs, IgM⁻/CD4⁺/CD25⁺; not shown).

Collection of Synchronized Cell Fractions

Centrifugal elutriation was done on New Zealand Black and non–New Zealand Black B-cell lines harvested at 4 to 8×10^5 cells/mL using the Beckman Coulter J6-MI, set at 2,700 rpm and 18°C. Cell fractions G₀/G₁ (G₁), late G₁/early S (G₁/S), S, late S/G₂ + M (S/G₂) were collected at flow speeds of 27, 30, 37, and 45 mL/min, as previously described (37). EDTA (1 mmol/L) in 1× PBS was used for the New Zealand Black cell line elutriation buffer, and 5 mmol/L EDTA in 1× PBS was used for the non–New Zealand Black cell line.

Transfection of Cells with miR-15a and miR-16 Mimics Asynchronous populations and elutriated fractions G₁, G₁/S, S, and S/G₂ were transfected using the Amaxa Nucleofector II instrument (Lonza Walkersville, Inc.) using programs L-013 (for BALB/c line), X-001 (for NFS/N line), and G-016 (for New Zealand Black line) with solutions V (for normal B lines) and T (for New Zealand Black cell line). Cells were transfected with 3 µg *miRIDIAN* Mimic mmumiR-15a, *miRIDIAN* Mimic mmu-miR-16, or *miRIDIAN* Mimic Negative Control 1 (a microRNA mimic-like duplex with a nonspecific sequence; Dharmacon), according to the manufacturer's protocol. Samples were plated at 5 to 6 × 10⁵ cells/mL and placed in a 37°C CO₂ incubator.

Cell Cycle Analysis

Cells (untreated, transfected, genistein treated) were stained with hypotonic propidium iodide (0.05 mg/mL propidium iodide, 0.1% Triton X-100, 0.1% sodium citrate) at either 24 or 48 h posttreatment, acquired on Becton Dickinson FACSCalibur using CELLQUEST software (Becton Dickinson), and analyzed using ModFit LT V3.1 software (Verity Software House).

MicroRNA Extraction and Quantification

Total RNA, including microRNA, was extracted from transfected and untransfected cells, according to the Trizol (Invitrogen) manufacturer's protocol. Quantitative realtime PCR was used to quantitate mature miR-16 expression from tissues and transfected samples using the TaqMan microRNA Reverse Transcription and TaqMan microRNA hsa-miR-16 Assay Kit (Applied Biosystems). The reverse transcription reaction was done using the GeneAmp PCR System 9600 (Perkin Elmer), and quantitative real-time PCR run on the Applied Biosystems 7500 Real-Time PCR Systems for 40 cycles at 60°C. A standard curve was generated using serial femtomole dilutions of miRIDIAN Mimic miR-16 (Dharmacon) to quantitate amount of miR-16 present before and posttransfection with miR-16. Real-time PCR was done to evaluate the expression of miR-16 in splenic cells isolated from New Zealand Black and normal mice using the TaqMan microRNA hsa-miR-16 Assay Kit, according to the manufacturer's protocol. The relative quantification values of New Zealand Black miR-16 levels compared with

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normal mouse strain miR-16 levels were determined using the standard $2^{-\Delta\Delta CT}$ method, according to the manufacturer's protocol. The total amount of input RNA was normalized to either Taqman 18S rRNA or U6 small nuclear RNA (Applied Biosystems).

Analysis of Cyclin D1 mRNA Levels

Total RNA was extracted from samples 24 h posttransfection with negative control or miR-16 mimic, as described above, and subjected to endpoint PCR to detect cyclin D1 mRNA levels. The reverse transcription step was executed



Figure 1. miR-16 expression is decreased in New Zealand Black B cells. A, spleen cells from 10 to 14 mo New Zealand Black and non-New Zealand Black strain mice were stained and sorted (three mice for each strain in three independent sorts). A representative sort from an individual New Zealand Black spleen. The initial population of CD4⁻ cells stained for IgM and B220 (CD45R; left) were sorted into conventional B-2 (IgM⁺, B220⁺; right top) and B-1 cells (IgM⁺, B220^{dull}; right bottom). B, real-time PCR was done on RNA extracted from each population. The relative quantification of miR-16 levels in New Zealand Black mice compared with non-New Zealand Black strain was determined using the standard $2^{-\Delta\Delta CT}$ method. miR-16 expression analysis in each population is reported relative to the expression in the non-New Zealand Black strain mouse B-2 population (normal strain B-2 = 1). (black columns, B-2; white columns, B-1 values). Error bars, the SEM for three independent , statistical significance between miR-16 levels of New Zealand sorts. Black B-1 and B-2 cells compared with C57 B-1 cells (P < 0.05).

using random hexamers and run on GeneAmp PCR System 9600 (Perkin Elmer). The cyclin D1 647-bp message was amplified on the GeneAmp 9600 at 60°C for 35 cycles using primers 5'-GCACACCTCTGGCTCTGTGC-3' (forward) and 5'-GCACCGGAGACTCAGAGC-3' (reverse), normalizing the amount of input RNA to hypoxanthine-guanine phosphoribosyltransferase. The following primers were used to distinguish cyclin D1a isoform from cyclin D1b (data not shown): 5'-CAGACCATCCGCAAGCATGC-3' (forward primer for D1a and D1b), 5'-CGACGTGCGAGATGTG-GAC-3' (reverse D1a), and 5'-GCATCAGGCAGACTGTGC-TAC-3' (reverse D1b). PCR products were then run on a 2% agarose gel and quantified using the Typhoon 9410 Variable Mode Imager (Amersham Biosciences) and ImageQuant 5.2 densitometric analysis software (Molecular Dynamics).

Analysis of Cyclin D1 Protein Levels

Predicted targets of miR-15a and miR-16, including cyclin D1, were obtained from TargetScan Whitehead Institute for Biomedical Research.⁴ The protein levels of target cyclin D1 were determined in transfected and untransfected cells (24 h posttransfection with miR-15a, miR-16, or negative control) by flow cytometric analysis. Cells were fixed in 2% paraformaldehyde and permeabilized with Triton X-100 (Sigma). Permeabilized cells were then stained with fluorescein isothiocyanate–conjugated anti-cyclin D1 (Biosource, Invitrogen). Single-color histogram samples were acquired on FACSCalibur (Becton Dickinson), collecting 2×10^4 events, and analyzed using CELLQUEST Software (Becton Dickinson).

Construction of Lentiviral Vector

A 647-bp fragment of the cyclin D1 3' UTR (chromosome 7: 3,185,751-3,186,397) containing three predicted miR-16 target sites according to TargetScan was cloned using the following primers: forward, 5'-GCACACCTCTGGCTCT-GTGC-3', and reverse, 5'-GCACCGGAGACTCAGAGC-3'. The sequence was then cloned into a bidirectional lentiviral vector in the 3' UTR of the green fluorescent protein (GFP) expression cassette. This vector, which has been described elsewhere (38), can be used as a sensitive reporter of micro-RNA activity (39). The lentivirus was produced using previously described methods (39, 40).

Lentivirus Sensor Assay

Our New Zealand Black malignant B-1 cell line was transduced separately with the Bd.LV.GFP vector lacking the cyclin D1 3' UTR sequence and with Bd.LV.GFP vector containing the cyclin D1 3' UTR sequence (because these are lentiviral vectors, they stably integrate into the genome of the cells). The transduced lines were then transfected with 3 μ g miR-15a, miR-16, or negative control (Dharmacon). Cells were incubated at 37°C, and flow cytometric analysis for GFP and monomeric Cherry (mCherry) expression was done at 24 h. One hundred thousand events per sample were acquired on the LSR II (BD Biosciences) and analyzed using FACSDiva Software (BD Biosciences). Five thousand events per sample were acquired on the Amnis ImageStream IS100

⁴ www.targetscan.org



Figure 2. Restoration of miR-15a and miR-16 results in G₁ arrest in New Zealand Black. Non-New Zealand Black and New Zealand Black B-cell lines were transfected with mimic negative control, miR-15a, or miR-16 and analyzed for cell cycle change 24 h posttransfection. Change in the amount of cells in G₁, S, and G₂ phases is a mean percent change of microRNA-transfected cells compared with those transfected with negative control. *Black columns*, percent change of cells in G₁; *gray columns*, S; *white columns*, G₂. The G₁ accumulation seen in the New Zealand Black B-cell line is significantly higher than that seen with the non-New Zealand Black B-cell line following miR-15a and miR-16 transfection. *, *P* < 0.008. The decrease in the amount of cells in S phase following transfection with miR-15a was significantly lower in the New Zealand Black B-cell line as compared with the non-New Zealand Black B-cell line. **, *P* < 0.02.

and analyzed using the Amnis IDEAS 3.0 software (Amnis Corporation).

Treatment of Cells with Nutlin and Genistein

Genistein and nutlin-3 (Sigma) were reconstituted in 100% dimethyl sulfoxide (DMSO) to make stock concentrations of 100 mmol/L and 1 mmol/L, respectively. Cells were untreated or treated with either 5 µmol/L nutlin-3 (Sigma) or 10 µmol/L genistein (Sigma; adding the drug directly to the media and the final concentration of DMSO being 0.01% or 0.5%), alone or immediately following transfection with 100 nmol/L miR-16 mimic or mimic negative control (Dharmacon). Cells were plated at 0.5×10^6 cells/mL and incubated at 37°C for 24 and 48 h. Dose response curves were generated by treating New Zealand Black B-cell line with varying doses of nutlin $(1-30 \mu mol/L)$ or genistein $(10-80 \mu mol/L)$. A suboptimal dose (at which apoptotic levels were between 10% and 15% of maximum apoptosis reached) was chosen for all subsequent treatments (Supplementary Fig. S1). DMSO-treated cells (0.01%, 0.5%) were also used as a control and were found to have no difference in apoptosis compared with untreated cells.

Statistical Analysis

All experiments were done in triplicate to obtain SDs and to calculate the SEM. Student's *t* test was used whereby appropriate to determine statistical significance, $P \le 0.05$.

Results

miR-16 Expression Is Decreased in Subpopulations of New Zealand Black B cells

Because the New Zealand Black spleen has decreased levels of miR-15a (data not shown) and miR-16 (14) as compared with non–New Zealand Black strain spleens, we wanted to further determine whether New Zealand Black malignant B-1 cells had a selective decrease in miR-16 expression. We analyzed sorted subpopulations containing nonmalignant B cells and malignant B cells. Spleen cells obtained from New Zealand Black and non-New Zealand Black strain mice (10-12 months of age) were sorted into conventional B-2 cells (CD4⁻, IgM⁺, B220⁺) or B-1 cells (CD4⁻, IgM⁺, B220^{dull}; Fig. 1A). RNA was obtained from the sorted B-cell populations, and the level of miR-16 expression was determined through real-time PCR. As anticipated, New Zealand Black conventional B-2 cells and B-1 cells (containing the malignant B clone) had less than half the amount of miR-16 expression relative to the B-2 and B-1 cell populations from age-matched non-New Zealand Black strain spleens (Fig. 1B). This indicates that all B cells from New Zealand Black mice are affected by the point mutation in the mir-15a/16-1 loci, resulting in decreased miR-16 expression.

Restoration of miR-16 Levels Results in G₁ Arrest in New Zealand Black–Derived Malignant B-1 Cell Line

In the New Zealand Black spleen, most B-2 cells are in G_{1} , whereas the malignant B-1 cells are cycling (14). To determine the effects of miR-16 on growth and cell cycle transit of these malignant B-1 cells, New Zealand Black B-1 and non-New Zealand Black B-cell lines were transfected with 3 µg of miR-15a or miR-16 mimic or a negative control mimic (because miR-16 shares a seed sequence and many predicted targets with miR-15a, we also transfected cells with a miR-15a mimic to test if both microRNAs would have the same effect on the cells). To observe cell cycle progression following transfection, cells were stained with hypotonic propidium iodide at 24 hours posttransfection and analyzed using ModFit LT V3.1 Software. New Zealand Black and non-New Zealand Black B-cell lines exhibited an increase in G₁ following transfection with miR-15a and miR-16 (similar to previous reports by our group and others; refs. 14, 24), yet G1 accumulation in the New Zealand Black B-cell line was significantly higher than that in the non-New Zealand Black B-cell line (Fig. 2). The New Zealand Black cell line also exhibited a significant decrease in S phase as compared with the non-New Zealand Black B-cell line (Fig. 2).

The New Zealand Black B-cell line was examined for any selective decrease in miR-16 specific to a cell cycle phase. Bcell lines were fractionated by cell cycle phase using centrifugal elutriation into the following fractions: fraction 1, early to mid- G_1 (G_1); fraction 2, late G_1 /early S (G_1 /S); fraction 3 (S); and fraction 4, late S to G_2/M (S/G₂). Centrifugal elutriation separates cells in a particular cell cycle phase on the basis of size, avoiding the artifacts associated with other cell synchronization techniques, such as altered gene expression (41). Representative results obtained following separation are shown (Fig. 3A). Analysis of basal levels of mature miR-16 expression revealed that the New Zealand Blackderived B-cell line had lower levels of miR-16 expression than did the non-New Zealand Black B-cell line at all stages of the cell cycle, with G₁ phase cells having significantly higher levels of miR-16 (Fig. 3B). Transfection with 3 μ g of miR-16 mimics resulted in an increase in the amount of

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microRNA miR-16 in all cell cycle phases following transfection in the non–New Zealand Black and the New Zealand Black B-cell lines (Fig. 3C). Cell cycle analysis was done on each transfected fraction 24 hours posttransfection by staining with hypotonic propidium iodide. In all elutriated fractions studied, New Zealand Black had greatly elevated G_1 arrest following miR-16 transfection when compared with the non–New Zealand Black B-cell line, with G_1 accumulation in the New Zealand Black B-cell line being significantly greater in fraction 4 (S/ G_2 ; Fig. 3D).

Restoration of miR-15a or miR-16 in the New Zealand Black–Derived Malignant B-1 Cell Line Correlates with a Decrease in Cyclin D1 Protein Levels

We hypothesized that the G_1 arrest observed in New Zealand Black cells upon transfection with miR-16 was a

result of the targeting of a cell cycle regulator by miR-16. TargetScan, a microRNA target prediction program, listed cyclin D1 as a likely target for miR-15a and miR-16 in humans and mice. To determine if cyclin D1 is regulated by miR-15a or miR-16, we transfected the New Zealand Black and non–New Zealand Black B-cell lines with either micro-RNA mimic or the negative control mimic and evaluated cyclin D1 mRNA and protein levels at 24 hours posttransfection. PCR revealed no change in cyclin D1 mRNA levels upon miR-16 addition, indicating that cyclin D1 was being regulated by miR-16 posttranscriptionally (Fig. 4A). In addition, PCR analysis revealed cyclin D1a as the dominant isoform in the New Zealand Black B-cell line (as compared with cyclin D1b isoform). Cyclin D1a mRNA includes all three predicted miR-15a/16 target sequences in its 3' UTR



Figure 3. Cell cycle regulated expression of miR-16. New Zealand Black and non-New Zealand Black B-cell lines were separated into cell cycle phase fractions by centrifugal elutriation. A, representative flow cytometric profiles of elutriated B-cell lines analyzed for cell cycle phase based on DNA content. In the four panels depicting each elutriated fraction: leftmost gray peak, the G_1 cells; rightmost gray peak, the G_2 cells; and hatched area, the S phase (by computer modeling). Elutriated fractions: G_1 (fraction 1), G_1/S (fraction 2), S (fraction 3), and S/G2 (fraction 4). B, basal levels of miR-16 expression in elutriated cells separated into the various phases of the cell cycle. Columns, the average miR-16 levels in femtomole, quantitated using real time PCR analysis. Black columns, non-New Zealand Black B-cell line; white, New Zealand Black B-cell line, miR-16 levels of cells in G1 are significantly lower in the New Zealand Black as compared with the non-New Zealand Black B-cell line. *, P < 0.04. **C**, the levels of miR-16 expression 24 h posttransfection of the elutriated fractions with either negative control or miR-16 mimic. Columns, mean values in femtomole (left, non-New Zealand Black; right, New Zealand Black). Black columns, cells transfected with negative control; white columns, with miR-16. Upon exogenous addition, miR-16 levels were significantly increased in the New Zealand Black B-cell line in G1,, G1/S, and S/G_2 . *, P < 0.03. **D**, the elutriated fractions were transfected with miR-16 or negative control for 24 h. Change in the amount of cells in G1 phase is the mean percent change of miR-16-transfected cells compared with those transfected with negative control. The G₁ accumulation seen in the New Zealand Black S/G₂ cell fraction 4 is significantly higher than G1 accumulation in the other New Zealand Black fractions. *, P ≤ 0.02. Error bars, SEM for at least three independent experiments.

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Figure 4. miR-15a and miR-16 regulate cyclin D1 posttranscriptionally. A, RNA was obtained 24 h posttransfection to observe the effects of miR-16 addition on levels of cyclin D1 mRNA. Bands, PCR products run on a 2% agarose gel (top). Mean ratio of densitometric analysis of CCND1 mRNA (647 bp) normalized to hypoxanthine-guanine phosphoribosyltransferase (162 bp; bottom). B, flow cytometry was used 24 h posttransfection to evaluate the protein levels of cyclin D1. Representative flow cytometric analysis of cyclin D1 expression in non-New Zealand Black B-cell line (top) and New Zealand Black B-cell line (bottom) after transfection with miR-16 (gray line) or negative control mimic (black line). The shaded histogram is the isotype control. Histograms are representative from four experiments. Percent decrease in mean fluorescence intensity of cyclin D1 protein levels 24 h after transfection with miR-16 as compared with negative control. C, schematic diagram of bidirectional lentiviral vector (top). A 647-bp portion of the cyclin D1 3' UTR containing three predicted miR-15 a/16 target sites was inserted downstream of GFP in a bidirectional lentiviral vector expressing GFP and mCherry (top). New Zealand Black B-cell line was stably transduced with Bd.LV.GFP.D1 (with cyclin D1 3' UTR) and Bd.LV.GFP (without 3' UTR) and transfected with miR-15a, miR-16, or negative control. Flow cytometry was used 24 h posttransfection to evaluate the expression levels of GFP and mCherry. Middle, representative Amnis images 24 h posttransfection (5,000 total cells analyzed per treatment group) of GFP, mCherry, or an overlay of GFP and mCherry expression in New Zealand Black B-cell line stably transduced with Bd.LV.GFP.D1 and transfected with miR-15a, miR-16, or negative control mimic (bright field image in far left). Bottom, mean percent change between negative control mimic and treated (miR-15a or miR-16) 24 h posttransfection of GFP expression relative to mCherry expression in individual cells analyzed by flow cytometry (100,000 cells per sample acquired on LSRII) in Bd.LV.GFP and Bd.LV.GFP.D1 transduced cell lines. *, significant decrease in GFP/mCherry expression compared with negative control mimic transfection (P < 0.001). Error bars, SEM for three independent experiments.

based on TargetScan (data not shown). Flow cytometric analysis did not detect a significant change in cyclin D1 protein expression in the non–New Zealand Black B-cell line but did detect a substantial decrease in the mean fluorescence intensity of cyclin D1 protein in the New Zealand Black B-cell line transfected with miR-16 compared with the negative control (Table 1; Fig. 4B). Transfection with miR-15a also resulted in a decrease in cyclin D1 protein levels in the New Zealand Black B-cell line (Table 1).

Cyclin D1 Is a Direct Target of miR-15a and miR-16

To confirm that miR-15a and miR-16 directly regulate cyclin D1, causing the observed decrease in protein levels, we cloned a 647-bp portion of the cyclin D1 3' UTR containing all three predicted miR-15a/16 target sites downstream

Table 1. Effect of transfection of microRNAs on cyclin D1 levels

Cell line	Treatment*	% Change cyclin D1
NZB	miR-16 miR-15a	-24.61 ± 6.38 -28.54 ± 4.41
non-NZB	miR-16 miR-15a	-5.55 ± 5.40 -14.31 ± 4.31

NOTE: Mean fluorescence intensity as compared with negative control. Abbreviation: NZB, New Zealand Black.

*Twenty-four hours posttransfection with 3µg negative control, miR-16, or miR-15a.

of the GFP expression cassette in a bidirectional lentiviral vector (Bd.LV.GFP.D1). The bidirectional lentiviral vector is an integrating vector that coordinately transcribes two transgenes, GFP and mCherry, as two distinct transcripts. Because the cyclin D1 3' UTR is contained in the GFP cassette, expression of mCherry is unaffected by posttranscriptional regulation of GFP and thus serves as an internal control (42). We separately transduced our New Zealand Black-derived malignant B-1 cell line with the Bd.LV.GFP vector lacking the cyclin D1 3' UTR sequence and with the Bd.LV.GFP.D1 vector containing the cyclin D1 3' UTR. Each stably transduced line was then transfected with miR-15a and miR-16 mimics and a negative control. As expected, transfection with the microRNA mimics did not suppress expression of GFP from the Bd.LV.GFP vector, as indicated by the ratio of the mean fluorescence intensity between GFP and mCherry in cells treated with the micro-RNA or negative control mimic. Instead, flow cytometric analysis showed that transfection of the miR-15a and miR-16 mimics resulted in a significant decrease in GFP expression, but not mCherry expression, in cells with the Bd. LV.GFP.D1 vector (Fig. 4C).

We then used Amnis ImageStream analysis to look at individual New Zealand Black cells transduced with Bd.LV. GFP.D1. Similar to the results from flow cytometry, we found that GFP and mCherry were expressed at similar levels in cells treated with the negative control mimic

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(Fig. 4C). However, treatment of these cells with either miR-15a or miR-16 decreased GFP expression, but not mCherry, with the overlay showing that a single cell produced mCherry but very little GFP (Fig. 4C). Together, these results show that miR-15a and miR-16 directly regulate cyclin D1 through target sites in the cyclin D1 3' UTR.

miR-16 Enhances the Ability of Nutlin and Genistein to Promote Apoptosis of Malignant B-1 Cells

To determine whether restoring miR-16 in the New Zealand Black B-cell line would enhance drug sensitivity, the effect of exogenous miR-16 plus a chemotherapeutic agent, such as nutlin and genistein, was investigated. Nutlin, a smallmolecule antagonist of MDM2, has been shown to activate p53 and induce apoptosis in cancer cells, including B-cell CLL (34). Genistein, an isoflavone derived from soybeans that also down-regulates cyclin D1, has also been shown to inhibit the growth of malignant cells while preserving normal cells (35). A dose-response curve was generated for nutlin and genistein, treating the New Zealand Black B-cell line with varying micromolar concentrations (Supplementary Fig. S1A and B). Suboptimal doses, at which the percentage of apoptotic cells were 10% to 15% of the maximum apoptotic level reached, were used to execute further experimentation in this report. We treated our New Zealand Black-derived malignant B-1 cell line and a non-New Zealand Black B-cell line with either 5 µmol/L of nutlin or 10 µmol/L of genistein alone and in conjunction with 100 nmol/L exogenous miR-16. Treating the New Zealand Black B-cell line with miR-16 and nutlin increased the percentage of cells in sub-G₁ (Fig. 5A), providing a synergistic effect, significantly enhancing apoptosis at 24 and 48 hours in the New Zealand Black B-cell line, but not in the non-New Zealand Black cell line (Fig. 5B). The addition of miR-16 significantly enhanced genistein-induced apoptosis



Figure 5. miR-16 enhances the apoptotic effects of nutlin and genistein on the New Zealand Black malignant B-1 cell line. The New Zealand Black B-cell line was treated with either 5 μ mol/L nutlin or 10 μ mol/L genistein alone or in conjunction with miR-16 or mimic negative control. Cells were stained with propidium iodide 24 and 48 h posttreatment to analyze DNA content and detect apoptosis levels. **A**, 24-h representative analysis of DNA content in untreated or treated (with nutlin and/or miR-16) New Zealand Black B- cell line. In each of the panels, the percentage of apoptotic cells is above the peak representing sub-G₁ (shaded peaks are computer modeled). **B**, As of the percentage of cells undergoing apoptosis in response to nutlin treatment, transfection with miR-16 or negative control, and nutlin + miR-16 or nutlin + negative control in non–New Zealand Black and New Zealand Black B-cell lines 24 and 48 h posttreatment. *Black columns*, difference in percent apoptosis between nutlin + miR-16 and nutlin + megative control. *Gray*, difference between nutlin + miR-16 or negative control. *C*, As of the percentage of apoptotic in non–New Zealand Black B not non–New Zealand Black B-cell lines 24 and 48 h posttreatment. *Black columns*, difference between mutlin + miR-16 or negative control, and genistein + miR-16 or genistein + negative control in non–New Zealand Black B and New Zealand Black B-cell lines 24 and 48 h posttreatment. *Black columns*, difference between miR-16 and negative control. *Gray*, difference between nutlin + miR-16 or genistein + negative control in non–New Zealand Black B. Cell lines 24 and 48 h posttreatment. *Black columns*, difference between miR-16 and negative control. *Gray*, difference between genistein + negative control in non–New Zealand Black B-cell lines 24 and 48 h posttreatment. *Black columns*, difference between miR-16 and negative control. *Gray*, difference between genistein + negative control in non–New Zealand Black B-cell lines 24 and 48 h posttreatment. *Blac*

in the New Zealand Black B-cell line at 24 and 48 hours, while having little to no effect in the non–New Zealand Black B-cell line, which had normal levels of miR-16 (Fig. 5C). At 48 hours, the combination treatment of miR-16 and genistein resulted in a significant synergistic induction of apoptosis (Fig. 5C).

Discussion

In this report, we found exogenous restoration of miR-16 to have cell cycle effects in the New Zealand Black B-1 cell line but little to no effect on non–New Zealand Black B-cell lines expressing normal levels of miR-16. The addition of miR-16 to the New Zealand Black B-cell line resulted in a G_1 arrest at all stages of the cell cycle and a decrease in cyclin D1 protein levels, as well as a sensitization to the apoptotic effects of nutlin and genistein.

The New Zealand Black B-1 cell line has decreased expression of miR-16 relative to the non–New Zealand Black B-cell line expression at all stages of the cell cycle. Because the alterations in the primary *mir-15a/16-1* loci were linked to the development of CLL in the New Zealand Black murine model (14), we investigated the role of miR-16 in cell cycle regulation and whether increasing miR-16 expression would alter the cell cycle and thereby malignant B-1 cell growth. In non–New Zealand Black and New Zealand Black B-cell lines, increasing miR-16 expression resulted in G₁ accumulation; however, the effect of miR-16 was much more pronounced in the New Zealand Black B-1 cell line. In addition, the cells in the late S and G₂/M (S/G₂) phase of the cell cycle were most sensitive to the effects of miR-16 transfection, showing the most G₁ accumulation.

One of the reported targets of miR-16 in human and mouse is cyclin D1 (32, 33). In the present report, reduced levels of miR-16 in the New Zealand Black correlates with the overexpression of the target gene CCND1 (cyclin D1). Among leukemias and lymphomas, elevated levels of cyclin D1 have primarily been associated with mantle cell lymphoma (29); however, reports have shown some CLL patients with high levels of cyclin D1 (30). Cyclin D1 and D3 levels have been reported to be higher in leukemic cells than in normal circulating B cells (43). In addition, increased levels of cyclin D1 were reported in CD5⁺ zap70⁺ CLL cells as compared with CD5⁺ zap70⁻ CLL cells (44), as well as in a subset of patients with clinically aggressive CLL (45). Our New Zealand Black-derived malignant B-1 cell line, LNC, mimics late-stage aggressive CLL (27). Deletions in the 3' UTR of cyclin D1 have been reported in CLL and are associated with overexpression of cyclin D1 (46, 47).

The New Zealand Black B cells have elevated cyclin D1 levels, which are similar to the elevated levels reported in some cases of human CLL. The previously reported mean fluorescence intensity ratio of cyclin D1 relative to isotype in patients with CLL had a range of 5.6 to 8.3 mean fluorescence intensity ratio (30), which is similar to that herein reported for untransfected New Zealand Black malignant B-1 cells (6.2 mean fluorescence intensity ratio of non-CLL normal control

individuals (4.8 mean fluorescence intensity ratio) was similar to the cyclin D1 levels following restoration of miR-16 in the New Zealand Black B-1 cells (4.9 mean fluorescence intensity ratio). Thus, exogenous miR-16 restored the level of cyclin D1 in the New Zealand Black B-cell line to a level similar to normal cells.

A selective effect of exogenous miR-16 on malignant cells, while sparing surrounding normal cells, has also been observed in nonlymphoid cancers resulting in G_1 arrest and decreased cyclin D1 (24, 26, 32, 33). We propose that decreased expression of cyclin D1, resulting from elevated miR-16 levels following transfection, played a significant role in the New Zealand Black B-cell line accumulation in G_1 . miR-16 was also shown to act synergistically with drugs nutlin and genistein to selectively increase the percentage of cells undergoing apoptosis in the New Zealand Black malignant B-cell line but not in the non–New Zealand Black B-cell line.

Our data support the involvement of microRNAs in cancer (48) and show miR-16 as a key regulator of cell cycle progression, G_1/S transition in particular. miR-16 may be used in a therapeutic approach to target and down-regulate oncogenic proteins overexpressed in cancers, such as cyclin D1. Restoration of miR-16 levels may also be used to increase apoptosis induced by other agents, increasing drug sensitivity in malignant cells deficient in miR-16.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Howard S. Mostowski from the Food and Drug Administration/ NIH for the cell sorting, Sukwinder Singh from University of Medicine and Dentistry of New Jersey for the help with the Amnis ImageStream, and the UMDNJ–New Jersey Medical School Flow Cytometry Core and Molecular Resource Facilities.

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Mol Cancer Ther 2009;8:2684-2692. Published OnlineFirst September 1, 2009.

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