MiR-15a and MiR-16 Control Bmi-1 Expression in Ovarian Cancer

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Abstract

Oncogenic activation of Bmi-1 is found in a wide variety of epithelial malignancies including ovarian cancer, yet a specific mechanism for overexpression of Bmi-1 has not been determined. Thus, realizing the immense pathologic significance of Bmi-1 in cancer, we wanted to investigate if microRNA (miRNA) aberrations played a role in the regulation of Bmi-1 in ovarian cancer. In this report, we identify two miRNAs, miR-15a and miR-16, that are underexpressed in ovarian cell lines and in primary ovarian tissues. We show that these miRNAs directly target the Bmi-1 3′ untranslated region and significantly correlate with Bmi-1 protein levels in ovarian cancer patients and cell lines. Furthermore, Bmi-1 protein levels are downregulated in response to miR-15a or miR-16 expression and lead to significant reduction in ovarian cancer cell proliferation and clonal growth. These findings suggest the development of therapeutic strategies by restoring miR-15a and miR-16 expression in ovarian cancer and in other cancers that involve upregulation of Bmi-1.

Introduction

Ovarian cancer is characterized by an initial response to cytotoxic chemotherapy, followed frequently by recurrence and disease progression, which represents a major scientific and clinical barrier to the control of cancer (1). Ovarian cancer has few symptoms early in its course, and therefore, the majority of patients are diagnosed with advanced-stage disease, which has a 5-year survival rate of only 20% to 25% (2, 3). Given this scenario, the development of new therapeutic strategies to combat ovarian cancer is needed.

Bmi-1 (B lymphoma mouse Moloney leukemia virus insertion region) plays a key role in regulating the proliferative activity of normal stem and progenitor cells (4). It is also indispensable for the self-renewal of neural (5) and hematopoietic stem cells (6). Overexpression of Bmi-1 has been reported in human non–small-cell lung cancer (7), breast cancer (8), prostate cancer (9), and, recently, ovarian cancer (10). Ovarian cancer tissues express high levels of Bmi-1, and the expression correlates with histologic grade and clinical phase of the disease. Also, overexpression of Bmi-1 causes neoplastic transformation of lymphocytes (11, 12). This suggests an oncogenic role for Bmi-1 activation in epithelial malignancies.

MicroRNAs (miRNA) are 21- to 23-nucleotide regulatory RNAs that control gene expression by targeting mRNA and triggering either translation repression or RNA degradation. The mammalian miRNAs have the potential to regulate at least 20% to 30% of all human genes (13). Emerging evidence suggests that altered regulation of miRNA is involved in the pathogenesis of many cancers (14). A number of studies have reported differentially regulated miRNAs in diverse cancer types (13, 15–21) including ovarian cancer (18, 22, 23). Collectively, these studies show that some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. Specific overexpression or underexpression of certain miRNAs has been shown to correlate with particular tumor types (24). Thus, realizing the immense pathologic significance of Bmi-1 in cancer, we wanted to investigate if miRNAs played any role in the regulation of Bmi-1 in ovarian cancer.

In this report, we identify two miRNAs, miR-15a and miR-16, which are underexpressed in ovarian cell lines and in primary ovarian tissues. We show that these miRNAs directly target the 3′ untranslated region (UTR) and significantly correlate with Bmi-1 protein levels in ovarian cancer patients and cell lines. Furthermore, Bmi-1 protein levels are downregulated in response to miR-15a or miR-16 expression and cause significant reduction in ovarian cancer cell proliferation and clonal growth. These findings suggest the development of therapeutic strategies by restoring miR-15a and miR-16 expression in ovarian cancer and in other cancers that involve upregulation of Bmi-1.

Materials and Methods

Reagents. [3H]Thymidine was from Amersham Biosciences. Phospholipase Cγ-1 antibody was from Santa Cruz Biotech., Inc., and Bmi-1 antibody was from Zymed.

Processing of ovarian cancer patient samples. Thirty-eight frozen optimal cutting temperature (OCT)–embedded ovarian cancer patient samples were provided by the Mayo Clinic Biospecimen Resource for Ovarian Cancer Research. These were then processed into five 10-μm sections each and suspended in 500 μl of TRizol reagent (Invitrogen). RNA and total protein were then isolated following the manufacturer’s protocol (Invitrogen).

Cell culture. OVCAR-5 cells were purchased from the American Type Culture Collection and grown in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycincycloheximide) according to the provider’s recommendation. OV-167, OV-202, and ovarian surface epithelial (OSE) cell lines were established and grown in MEM supplemented with 10% and 20% FBS, respectively, and 1% antibiotic as previously described (25). CP-70 and A2780 cell lines were grown in RPMI supplemented with 10% FBS and 1% antibiotics.

miRNA transfection. The ovarian cell lines, OVCAR-5, OV-167, CP-70, A2780, or OV-202, were grown in their respective medium 1 dB transfecion. Using Oligofectamine, the cells were transfected with 50 nM/mL of negative control 1 precursor miRNA, miR-15a, or miR-16. After 48 h, the cells were processed for Western blot, proliferation, or clonogenic growth assays. For the Bmi-1 rescue experiments, the ovarian cancer cells were transfected with 50 nM/mL miRNA along with 1 μg of Bmi-1 construct using Lipofectamine Plus (Invitrogen). After 48 h, the cells were lifted and plated in 24-well plates for proliferation assays.

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

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Western blot. Harvested ovarian cancer cells, both treated and nontreated, were washed in PBS and lysed in ice-cold radioimmunoprecipitation assay buffer with freshly added 0.01% protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. Cell debris was discarded by centrifugation at 13,000 rpm for 10 min at 4°C and the supernatant (30–50 μg of protein) was run on an SDS-PAGE gel (25).

Cell proliferation assay. Ovarian cells (2 × 10^4) were seeded in 24-well plates and cultured for 48 h. Subsequently, 1 μCi of [3H]thymidine was added; 4 h later, cells were washed with chilled PBS, fixed with 100% cold methanol, and collected for measurement of trichloroacetic acid–precipitable radioactivity. Experiments were repeated at least three times each time in triplicate, and assay done as previously described (25).

Clonogenic assays. After miRNA transfection, clonogenic assays were done as follows: OV-167 and CP-70 cells were plated in 60-mm plates at 200 cells/mL. Colonies were counted after staining the cells with 0.1% crystal violet within 7 to 10 d after plating (25).

Vector construction. Two different interaction sites were predicted for miR-15a, positions 3002–3023 and 3108–3129 on the Bmi-1 3′UTR (NM_005180.5). The second site, position 3108–3129, was also predicted for miR-16 interaction. Two different constructs were prepared by deleting 8 bp of the predicted miR interaction sites in the Bmi-1 3′UTR luciferase reporter construct obtained from Stratagene. Mutant #1 had the miR-15a site deleted and was constructed using the following primers: 5′-gctttgtcttgctatatgtaattatatcataaactatatttcatatactctcctgctttataaaatag-3′ and 3′-cataatattttaaagcgaagagaataatgtaaagttatggacaagc-5′. Mutant #2 had the common site for both miR-15a and miR-16 deleted using the following primers: 5′-gctttgtcttgctatatgtaattatatcataaactatatttcatatactctcctgctttataaaatag-3′ and 3′-cataatattttaaagcgaagagaataatgtaaagttatggacaagc-5′.

Luciferase reporter assays. Five thousand ovarian cancer cells were plated in 96-well plates. After 24 h, the cells were transfected with 50 nmol/L mirRNA (final concentration) along with the wild-type Bmi-1 3′UTR luciferase construct or mutant #1 or mutant #2 (final concentration, 100 ng/well) as described above. Forty-eight hours after transfection, luciferase activity was measured using SteadyGlo assay system (Promega). Luciferase activity was calculated as follows: Light output with mirR-15a or mirR-16 mimic / Light output with negative control 1 precursor miRNA = Activity of mirR-15a or mirR-16 on target 3′UTR.

RNA extraction, retrotranscription, and real-time PCR. Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen). For quantification of transfected and/or endogenous mature mirRNA levels, TaqMan MicroRNA assays (Applied Biosystems) were used. RNA was first retrotranscribed with mirRNA-specific primers using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and then real-time PCR was carried out using TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). The comparative Ct method was used to calculate the relative abundance of miRNA compared with RNU6B expression (fold difference relative to U6; ref. 26).

Statistical analysis. All values are expressed as means ± SD. Statistical significance was determined using two-sided Student’s t test, and P < 0.05 (*) was considered significant.

Results

Bmi-1 is overexpressed in ovarian cancer. Recent experimental observations documented increased expression of Bmi-1 in human non–small-cell lung cancer (7), breast carcinoma (8), prostate carcinoma (9), and in 80.9% of the cases in ovarian cancer (10), suggesting an oncogenic role for Bmi-1 in the progression of epithelial malignancies. In accordance with our previous observation (25), we confirmed that expression of Bmi-1 was higher in a panel of ovarian cancer cell lines tested when compared with nonmalignant OSE cells (Fig. L4). Comitantly, expression of Bmi-1 was determined by Western blot in a panel of 38 high-grade serous ovarian cancer samples. According to the relative expression of Bmi-1 (with respect to β-actin), ovarian cancer samples were divided into high and low Bmi-1 expressing groups (Supplementary Fig. S1). The band intensities of β-actin and Bmi-1 from each patient were determined by NIH Image densitometry (26). If the Bmi-1/β-actin ratio was >0.7, it was considered to be high, and if the ratio was <0.7, it was considered to be low (Fig. 1B). Of the 38 samples, 10 showed low and 28 showed high expression of Bmi-1, thus suggesting an important role of Bmi-1 in the pathogenesis of ovarian cancer.

Prediction of putative miRNAs interacting with Bmi-1. Possible miRNAs targeting Bmi-1 were found using the MirGen prediction integrated database of animal miRNA targets (27). Articles related to ovarian cancer (18, 23, 28) were also manually analyzed to extract miRNAs downregulated in ovarian cancer samples. By selecting only for miRNAs that were able to target Bmi-1 and were simultaneously lost in ovarian cancer, we identified miR-15a and miR-16 as potential targets for Bmi-1.

MiR-15a and miR-16 levels correlate with Bmi-1 expression in ovarian cancer. Knowing that Bmi-1 is overexpressed, we next sought to determine the expression levels of miR-15a and miR-16 in our panel of ovarian cancer samples. First, we determined by reverse transcription-PCR (RT-PCR) the expression levels of miR-15a and miR-16 in six ovarian cell lines. MiR-15a and miR-16 relative levels (with respect to RNU6B; see Materials and Methods) were found to be lower for each of the ovarian cancer cell lines tested in comparison with control OSE cells (Fig. 2A).

Subsequently, we evaluated miR-15a and miR-16 expression levels in the 38 ovarian cancer patient samples (Supplementary Fig. S2) and correlated these data with Bmi-1 expression levels obtained previously from these same samples (Fig. 1B). We observed that ovarian cancer samples characterized by high Bmi-1 protein levels presented low levels of both miR-15a and miR-16, and that low Bmi-1 expressing samples had instead high levels of miR-15a

http://www.diana.pcbi.upenn.edu/miRGen.html
and miR-16. The differences in miRNA expression observed between high and low Bmi-1 expressing samples were statistically significant ($P = 0.04$ and $P = 0.02$ for miR-15a and miR-16, respectively; Fig. 2B). These data suggested that a clear inverse correlation existed between miR-15a and miR-16 levels and Bmi-1 expression.

**Bmi-1 is a direct target of miR-15a and miR-16.** In comparison with negative control 1 precursor miRNA, transfection of miR-15a and miR-16 into ovarian cell lines caused a significant decrease in protein levels of Bmi-1 as determined by Western blot (Fig. 3). Similarly, the Bmi-1 3′UTR (OV-202 and CP-70) luciferase activity was also significantly lower in the miR-15a- and miR-16-transfected cells compared with the control (Fig. 4). Mutation of the single miR-16 site rescued the luciferase activity, thus confirming a direct interaction of miR-16 with the 3′UTR of Bmi-1 mRNA. However, mutation of the two miR-15a sites independently rescued the luciferase activity partially (Fig. 4). Therefore, two possibilities exist: The double mutant could completely rescue inhibition of luciferase activity, or other interaction sites for miR-15a might exist in the 3′UTR of Bmi-1. Thus, after determining that miR-15a and miR-16 target the 3′UTR of Bmi-1 as well as downregulate Bmi-1 protein levels, we next wanted to determine the effect of these
miRNAs on the proliferation and clonal growth of ovarian cancer cells.

**MiR-15a and miR-16 control the proliferation and clonal growth of ovarian cell lines.** According to our and others’ previous observations, Bmi-1 is overexpressed in ovarian cancer cell lines as well as in primary tissues. Bmi-1 is also known to regulate proliferative and clonal capacity in a number of different cell types. Therefore, we hypothesized that restoration of miR-15a or miR-16 in ovarian cancer cells, via Bmi-1 downregulation, could affect cell proliferation. To this end, we performed proliferation and clonal growth assays after overexpressing miR-15a or miR-16 in the ovarian cancer cell lines. The cells were transfected with negative control 1 precursor miRNA, miR-15a, or miR-16 for 48 hours. Proliferation was measured by \[^{3}H\]thymidine incorporation assay 48 hours after plating the miRNA-transfected cells into 24-well plates. In the three cell lines tested, OV-167, OV-202, and CP-70, a significant decrease in proliferation was observed in miR-15a− or miR-16− transfected cells compared with the control (Fig. 5A). Similar results were obtained in the clonal growth assay (Fig. 5B). To further confirm that the miRNA induced inhibition in proliferation was specifically due to the downregulation of Bmi-1 through its 3′UTR, we overexpressed the miRNAs along with a Bmi-1 construct without some of the 3′UTR and thus unresponsive to inhibition by the miRNA. As expected, overexpression of the Bmi-1 construct rescued the decreased proliferation phenotype of the miR-15a− and miR-16− transfected OV-202 cells (Fig. 5B). Similar results were obtained with OV-167 and CP-70 (data not shown). These results prove that in ovarian cancer, miR-15a and miR-16 indeed regulate proliferation and clonal growth through downregulation of Bmi-1.

**MiR-15a and miR-16 also target Bcl-2 in ovarian cancer cell lines.** In a previous study, miR-15a and miR-16 were reported to suppress Bcl-2, an antiapoptotic gene in B-cell chronic lymphocytic leukemia (29). Therefore, to determine whether the effects of restoration of miR-15a or miR-16 were via downregulation of Bmi-1 or Bcl-2 in ovarian cancer cells, we first determined the expression of endogenous Bcl-2 by Western blot. In a panel of ovarian cancer cell lines, the expression of Bcl-2 was very low except in the OV-202 cell line (Fig. 6A). Subsequently, we also confirmed the downregulation of Bcl-2 levels in OV-202 cells on restoration of miR-15a or miR-16 (Fig. 6B). Bcl-2 is a known inhibitor of apoptosis and its effect, if any, on the proliferation and clonal growth of ovarian cells is not clear. Furthermore, rescuing Bmi-1 expression through a construct nonresponsive to miR-15a or miR-16 restores proliferation in OV-202 cells (Fig. 5B). Therefore, the effects of Bcl-2 downregulation on proliferation, if any, are minimal.

**Discussion**

miRNAs have recently been described as important players in human cancer and their role as therapeutic targets has been proposed. The expression of miRNAs is remarkably deregulated in ovarian cancer, strongly suggesting that miRNAs are involved in the initiation and progression of this disease (30). In this study, we have identified the transcriptional repressor Bmi-1 as a target for miR-15a and miR-16 in ovarian cancer.

![Figure 4](https://example.com/f4.png)

**Figure 4.** Targeting of the Bmi-1 3′UTR by miR-15a and miR-16. The ovarian cancer cell lines were transfected either with the wild-type Bmi-1 3′UTR construct bearing reporter luciferase or with two mutants bearing deletions at predicted miR-15a or miR-16 interaction sites along with negative control 1 precursor miRNA (control miR), miR-15a, or miR-16 using Lipofectamine Plus reagent. After 48 h, luciferase activity was determined using SteadyGlo (Promega). *, *P* < 0.05.
The Bmi-1 gene is widely expressed in diverse human tumors, including lymphomas, non–small cell lung cancer, B-cell non-Hodgkin’s lymphoma, breast cancer, colorectal cancer, and neuroblastoma, and has been shown to be a useful prognostic marker in myelodysplastic syndrome and many cancers, including nasopharyngeal carcinoma and gastric cancer. In accordance with a previous publication that reported high expression of Bmi-1 in 80.9% of the ovarian cancer cases, our Western blot studies showed high expression levels in ∼74% and low expression levels of Bmi-1 in ∼26% of the patient samples, thus suggesting an important role for Bmi-1 in ovarian cancer (10).

In a recent study, Bmi-1 was targeted by miR-128 in glioma cells and shown to regulate self-renewal (31). However, we found that levels of miR-128 did not significantly differ between OSE and other ovarian cancer cell lines, except in A2780 where it was higher (Supplementary Fig S3). These results suggest that miR-128, at least in ovarian cancer, does not play an important role in the regulation of Bmi-1. The first study documenting abnormalities in miRNA expression in tumors identified miR-15a and miR-16, which were located in a frequently deleted region in B-cell chronic lymphocytic leukemia (32). MiR-15a and miR-16 were also expressed at lower levels in pituitary adenomas as compared with normal pituitary tissue (33). We found that miR-15a and miR-16 were downregulated in the ovarian cancer cell lines and in the patient samples. Furthermore, both in ovarian cancer cell lines and in patient samples, a strong inverse correlation exists between Bmi-1 expression and miR-15a and miR-16 levels. In accordance, at least four different studies reported significant downregulation of miR-15a and miR-16 in ovarian tumors, and these were associated with genomic copy number loss or epigenetic silencing or were due to compromised miRNA processing machinery such as the reduced expression of Dicer (23, 30, 34, 35). However, the significance of this downregulation was not clear. Our data showing inhibition of proliferation and clonal growth of ovarian cancer cells on expression of miR-15a or miR-16 suggest that downregulation of miR-15a or miR-16 may contribute to ovarian tumor growth by regulating Bmi-1 protein levels.

The pertinent question that we ask is “What are the targets that are responsible for the miR-15a– or miR-16–induced decrease in the proliferation and clonal growth of ovarian cancer cells?” MiRNAs including miR-15a or miR-16 can affect hundreds of mRNAs, which renders it difficult to identify the biologically relevant targets. However, we were able to show that miR-16 specifically interacted with the 3’ UTR of Bmi-1 and regulated its expression levels. Furthermore, mutation of the two miR-15a sites independently rescued luciferase activity only partially. Therefore, two possibilities might explain such results: (a) Double mutant could completely rescue inhibition of luciferase activity, or (b) other interaction sites for miR-15a exist in the 3’UTR of Bmi-1.

In ovarian cancer, regulators of Bmi-1 expression are likely to be critical determinants of proliferation, clonal growth, self-renewal, and even chemoresistance (36). Physiologically, miR-15a and miR-16 exert their effects through action on multiple targets such as Bcl-2.

![Figure 5](image1.png) **Figure 5.** Effect of miRNA expression on the proliferation and clonal growth of ovarian cancer cell lines. A, overexpression of miR-15a or miR-16 causes significant inhibition of clonal growth in ovarian cancer cell lines. B, overexpression of miR-15a or miR-16 causes significant inhibition of proliferation in ovarian cancer cell lines. Overexpression of miR-15a or miR-16 along with a Bmi-1 construct unresponsive to miRNA rescues the inhibition in proliferation of OV-202 cells. *, P < 0.05 was considered significant.

![Figure 6](image2.png) **Figure 6.** Expression of Bcl-2 in ovarian cancer cell lines. A, cell lysates from different ovarian cancer cells were run on an SDS-PAGE gel and subjected to Western blot using Bcl-2 antibody. β-Actin was used as a loading control. B, OV-202 cells were transfected with control miRNA, miR-15a, or miR-16 using Oligofectamine. After 48 h, cell lysates were collected and run on an SDS-PAGE gel for Western blotting with Bcl-2 and β-actin antibodies.
miRNAs Modulate Ovarian Cancer via Regulation of Bmi-1

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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