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MiR-15a and MiR-16 control Bmi-1 expression in ovarian cancer

Resham Bhattacharya^{1,*}, Milena Nicoloso², Rochelle Arvizo¹, Enfeng Wang¹, Angelica Cortez², Simona Rossi², George A Calin², and Priyabrata Mukherjee^{1,4}

¹Department of Biochemistry and Molecular Biology, College of Medicine, Mayo Clinic, Rochester, 55905, USA

²Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center

³Department of Urology, College of Medicine, Mayo Clinic, Rochester, 55905, USA

⁴Department of Biomedical Engineering, College of Medicine, Mayo Clinic, Rochester, 55905, USA

Abstract

Oncogenic activation of Bmi-1 is found in a wide variety of epithelial malignancies including ovarian cancer, yet a specific mechanism for over expression of Bmi-1 has not been determined. Thus realizing the immense pathological significance of Bmi-1 in cancer, we wanted to investigate if microRNA aberrations played a role in the regulation of Bmi-1 in ovarian cancer. In this report we identify two microRNAs, miR-15a and miR-16 that are under expressed in ovarian cell lines and in primary ovarian tissues. We demonstrate that these miRNAs directly target the Bmi-1 3' UTR and significantly correlate with Bmi-1 protein levels in ovarian cancer patients and cell lines. Furthermore, Bmi-1 protein levels are down regulated 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 up regulation of Bmi-1.

Keywords

MicroRNA; ovarian cancer; Bmi-1; clonal growth; proliferation

Introduction

Ovarian cancer is characterized by an initial response to cytotoxic chemotherapy, followed frequently by recurrence and disease progression that 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 haematopoietic stem cells (6). Over expression of Bmi-1 has been reported in human non-small-cell lung cancer (7), breast

*Address for Correspondence: Dr. Resham Bhattacharya, Department of Biochemistry and Molecular Biology, College of Medicine, Mayo Clinic Rochester, MN-55905, USA, Phone: 507-538-0563, Fax: 507-284-1767, bhattacharya.resham@mayo.edu.

cancer (8), prostate cancer (9) and recently in ovarian cancer (10). Ovarian cancer tissues express high levels of Bmi-1 and expression correlates with histological grade and clinical phase of the disease. Also over expression of Bmi-1 causes neoplastic transformation of lymphocytes (11,12). This suggests an oncogenic role for Bmi-1 activation in epithelial malignancies.

Micro RNAs (miRNAs) are 21–23 nucleotide regulatory RNAs that control gene expression by targeting messenger RNA and triggering either translation repression or RNA degradation. The mammalian miRNAs have the potential to regulate at least 20–30% of all human genes (13). Emerging evidence suggests that altered regulation of microRNA 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 demonstrate that some human microRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. Specific over or under expression of certain microRNAs has been shown to correlate with particular tumor types (24). Thus, realizing the immense pathological significance of Bmi-1 in cancer, we wanted to investigate if microRNAs played any role in the regulation of Bmi-1 in ovarian cancer.

In this report we identify two microRNAs, miR-15a and miR-16 that are under expressed in ovarian cell lines and in primary ovarian tissues. We demonstrate that these miRNAs directly target the 3' UTR and significantly correlate with Bmi-1 protein levels in ovarian cancer patients and cell lines. Furthermore, Bmi-1 protein levels are down regulated 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 up regulation of Bmi-1.

Material and methods

Reagents

[³H]thymidine was from Amersham Biosciences. PLCγ-1 antibody was from Santa Cruz Biotech. Inc., CA and Bmi-1 antibody was from Zymed, CA, USA.

Processing of ovarian cancer patient samples

38 ovarian cancer, frozen-OCT patient samples were provided by the Mayo Clinic Biospecimen Resource for Ovarian Cancer Research. These were then processed into five 10-micron sections each and suspended in 500 µl TRIzol reagent (Invitrogen). RNA and total protein were then isolated using manufacturer's protocol (Invitrogen).

Cell Culture

OVCAR-5 cells were purchased from ATCC and grown in DMEM with 10% FBS and 1% antibiotic (Penicillin/Streptomycin) according to the provider's recommendation. OV-167, OV-202 and 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 cells were grown in RPMI supplemented with 10% FBS and 1% antibiotics.

MicroRNA transfection

The ovarian cells, OVCAR-5, OV-167, CP-70, A2780 or OV-202 were grown in their respective medium for one day prior to transfection. Using oligofectamine the cells were transfected with 50nM negative control 1 precursor miR or miR-15a or miR-16. After 48hrs. 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 microRNA along with 1 µg Bmi-1 construct using Lipofectamine Plus (Invitrogen). After 48hrs. the cells were lifted and plated in 24-well plates for proliferation assays.

Western Blot

Harvested ovarian cancer cells, both treated and non-treated, were washed in PBS and lysed in ice-cold radioimmunoprecipitation (RIPA) buffer with freshly added 0.01% protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. Cell debris was discarded by centrifugation at 13000 rpm for 10 min at 4°C and the supernatant (30–50 µg of protein) was run on an SDS-Page (25).

Cell Proliferation Assay

Ovarian cells (2×10^4) were seeded in 24-well plates and cultured for 48hrs. Subsequently 1 µCi of [^3H]thymidine was added; 4h 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 performed as previously described (25).

Clonogenic Assays

Following microRNA transfection clonogenic assays were performed as follows; OV-167, and CP-70 cells were plated in 60mm plates at 200 cells per ml respectively. Colonies were counted after staining the cells with 0.1% crystal violet within 7–10 days after plating (25).

Vector construction

Two different interaction sites were predicted for miR-15a, position 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 Siwthgear Genomics using the QuickChange kit (Stratagene). Mutant#1 had the miR-15a site deleted and was constructed using the following primers: (5'-3') gcttgctctgcttatagtcattaaatcattactttacatatatctctgcttcttataaaatag and (3'-5') ctatattttaaagaaagcagaagatatgtaaaagtaagtatttaagtactataagcaagacaaagc. Mutant#2 had the common site for both miR-15a/16 deleted using the following primers: (5'-3') gacctaatttgtagctccattgtaattctaattatagatgtaaaatgaaatttc and (3'-5') gaaatttcattttacatctataaattagaattacaatgggactgtacaaatttaggtc.

Luciferase reporter Assays

5000 ovarian cancer cells were plated in 96-well plates. After 24hrs. the cells were transfected with 50nM microRNA (final concentration) along with the wild-type Bmi-1 3'UTR-luciferase construct or mutant#1 or mutant#2 (100 ng /well final concentration) as described above. 48hrs. after transfection luciferase activity was measured using SteadyGlo assay system (Promega). Luciferase activity was calculated as follows: Light output with miR-15a or miR-16 mimic/Light output with negative control 1 precursor miR = Activity of miR-15a or miR-16 on target 3'UTR.

RNA extraction, Retrotranscription and Realtime PCR

Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen). For quantification of transfected and/or endogenous mature microRNA levels TaqMan® MicroRNA assays (Applied Biosystems) were used. RNA was first retrotranscribed with microRNA specific primers using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and then realtime PCR was carried out using and TaqMan® Universal PCR

Master Mix, No AmpErase® UNG (Applied Biosystems). The comparative C_t method was used to calculate the relative abundance of microRNA compared with RNU6B expression (Fold difference relative to U6) (26).

Statistical analysis

All values are expressed as means $\pm$ SD. Statistical significance was determined using two-sided Student's *t* test, and a value of $P < 0.05$ (*) was considered significant.

Results

Bmi-1 is over expressed 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 to nonmalignant ovarian surface epithelial cells (OSE) (Fig 1A). Concomitantly, 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 beta-actin), ovarian cancer samples were divided into high and low Bmi-1 expressing groups (Supplement to Fig 1B). The band intensity of beta-actin and Bmi-1 from each patient was determined by NIH Image densitometry. If the Bmi-1:beta-actin ratio was >0.7 it was considered to be high and if <0.7 was considered to be low (Fig 1B). Of the 38 samples, 10 were 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 microRNAs interacting with Bmi-1

Possible microRNAs targeting Bmi-1 were found using the MiRGen prediction integrated database of animal miRNA targets (27) ([†]), according to combinations of widely used target prediction programs. Ovarian cancer related papers (18,23,28) were also manually analyzed to extract miRNAs down regulated 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 the Bmi-1.

MiR-15a and miR-16 levels correlate with Bmi-1 expression in ovarian cancer

Knowing that Bmi-1 is over expressed 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 RT-PCR miR-15a and miR-16 expression levels in 6 ovarian cell lines. MiR-15a and miR-16 relative levels (with respect to RNU6B, see methods), were found to be lower for each of the ovarian cancer cell lines tested in comparison to control OSE cells (Fig 2A).

Subsequently, we evaluated miR-15a and miR-16 expression levels in the 38 ovarian cancer patient samples (Supplement to Fig 2B), and correlated this 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 and miR-16. The differences in microRNA expression observed between high and low Bmi-1 expressing samples were statistically significant ($p=0.04$ and $p=0.02$ for miR-15a

[†]<http://www.diana.pcbi.upenn.edu/miRGen.html>

and miR-16 respectively) (Fig 2B). This 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 microRNA, 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 to 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, one that 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 down regulate Bmi-1 protein levels, we next wanted to determine the effect of these microRNAs on proliferation and clonal growth of ovarian cancer cells.

MiR-15a and miR-16 control proliferation and clonal growth of ovarian cell lines

According to our and other's previous observations Bmi-1 is over expressed 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 down regulation, could affect cell proliferation. To this end we performed proliferation and clonal growth assays after over expressing miR-15a or miR-16 in the ovarian cancer cell lines. The cells were transfected either with the negative control 1 precursor miRNA or miR-15a or miR-16 for 48 hrs. Proliferation was measured by [³H]-thymidine incorporation assay 48 hrs. after plating the miRNA transfected cells into 24-well plates. In the three cell lines tested, OV-167, OV-202 and CP-70 significant decrease in proliferation was observed in miR-15a or miR-16 transfected cells compared to the control (Fig 5A). Similar results were obtained in the clonal growth assay (Fig 5B). To further confirm that the microRNA induced inhibition in proliferation was specifically due to the down regulation of Bmi-1 through its 3'UTR, we over expressed the miRNAs along with a Bmi-1 construct without some of the 3'UTR and thus non-responsive to inhibition by the microRNA. As expected over expression 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 down regulation 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 in order to determine whether the effects of restoration of miR-15a or miR-16 were via down regulation 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 down regulation of Bcl-2 levels in OV-202 cells upon restoration of miR-15a or miR-16 (Fig 6B). Bcl-2 is a known inhibitor of apoptosis and its effect if any on proliferation and clonal growth of ovarian cells is not clear. Furthermore rescuing Bmi-1 expression through a construct non-responsive to miR-15a or miR-16 restores proliferation in OV-202 cells (Fig 5B). Therefore effects of Bcl-2 down regulation 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.

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 demonstrated 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 (Supplement to Fig 2A). These results suggest that miR-128, at least in ovarian cancer, does not play an important role in regulating 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 to normal pituitary tissue (33). We found that miR-15a and miR-16 were down regulated 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 down regulation 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 microRNA processing machinery such as the reduced expression of Dicer (23,30,34,35). However, the significance of this down regulation was not clear. Our data demonstrating inhibition of proliferation and clonal growth of ovarian cancer cells upon expression of miR-15a or miR-16 suggest that down-regulation of miR-15a or miR-16 may contribute to ovarian tumor growth by regulating Bmi-1 protein levels.

The pertinent questions that we ask is what are the targets that are responsible for miR-15a or miR-16-induced decrease in proliferation and clonal growth in ovarian cancer cells? MicroRNAs 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 demonstrate 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; i) double mutant could completely rescue inhibition of luciferase activity or ii) 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 chemo-resistance (36). Physiologically, miR-15a and miR-16 exert their effects through action on multiple targets such as Bcl-2 (29). However, in ovarian cancer, miR-15a and miR-16 consistently down regulate Bmi-1 and affect proliferation and clonal growth thus suggesting their potential as therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1A **Fig 1B** **Figure 1.**

Expression of Bmi-1 in ovarian cancer cell lines and patient samples. (A) Cellular lysates from different ovarian cancer cells were run on an SDS-Page gel and subjected to Western blot using Bmi-1 antibody. Beta actin was used as a loading control. (B) Protein was collected from 38 frozen-OCT patient samples using the TRIzol method and subjected to western blot using Bmi-1 antibody. Beta actin was used as a loading control. In OV9 band intensity of Bmi-1 as determined by NIH Image densitometry / band intensity of beta actin=1. In OV10 band intensity of Bmi-1 / band intensity of beta actin=0.41. A representative image is shown here. Such ratios were calculated for all the 38 samples and the Bmi-1:beta-actin ratio >0.7 was considered to be high and if <0.7 was considered to be low expressor.

Fig 2A

Fig 2B

Figure 2.

Expression of miR-15a and miR-16 and correlation with Bmi-1 levels in ovarian cancer cell lines and patient samples. (A) Total RNA was collected from the ovarian cancer cell lines using the TRIzol method and subjected to RT-PCR. The comparative C_t method was used to calculate the relative abundance of miR-15a or miR-16 with respect to RNU6B expression. Relative fold difference in microRNA with respect to OSE cells is represented. $P < 0.05$ (*) was considered significant. (B) The average relative fold difference for miR-15a and miR-16 were calculated and plotted against the high versus low Bmi-1 expressor groups from the patient samples. The differences in microRNA 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).

Figure 3. Effect of microRNA expression on Bmi-1 protein levels in ovarian cancer cell lines. The ovarian cancer cell lines were transfected with the negative control 1 precursor miR (SC), miR-15a or miR-16 using oligofectamine. After 48hrs. cell lysates were collected and run on an SDS-Page for western blotting with Bmi-1 and PLCγ-1 antibody.

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 the negative control 1 precursor miR, miR-15a, or miR-16 using Lipofectamine Plus reagent. After 48hrs. luciferase activity was determined using SteadyGLO (Promega). $P < 0.05$ (*) was considered significant.

Figure 5.

Effect of microRNA expression on proliferation and clonal growth of ovarian cancer cell lines. (A) Over expression of miR-15a or miR-16 causes significant inhibition of clonal growth in ovarian cancer cell lines. (B) Over expression of miR-15a or miR-16 causes significant inhibition of proliferation in ovarian cancer cell lines. Over expression of miR-15a or miR-16 along with a Bmi-1 construct non-responsive to microRNA rescues the inhibition in proliferation OV-202 cells.

Figure 6.

Expression of Bcl-2 in ovarian cancer cell lines. (A) Cellular lysates from different ovarian cancer cells were run on an SDS-Page gel and subjected to Western blot using Bcl-2 antibody. Beta actin was used as a loading control. (B) OV-202 cells were transfected with the control, miR-15a or miR-16 using oligofectamine. After 48hrs. cell lysates were collected and run on an SDS-Page for Western blotting with Bcl-2 and beta actin antibody.