Restoration of miR-200c to Ovarian Cancer Reduces Tumor Burden and Increases Sensitivity to Paclitaxel

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Abstract

A therapeutic intervention that could decrease tumor burden and increase sensitivity to chemotherapy would have a significant impact on the high morbidity rate associated with ovarian cancer. miRNAs have emerged as potential therapeutic candidates due to their ability to downregulate multiple targets involved in tumor progression and chemoresistance. miRNA-200c (miR-200c) is downregulated in ovarian cancer cell lines and stage III ovarian tumors, and low miR-200c correlates with poor prognosis. miR-200c increases sensitivity to taxanes in vitro by targeting class III β-tubulin gene (TUBB3), a tubulin known to mediate chemoresistance. Indeed, we find that patients with tumors having low TUBB3 had significantly prolonged survival (average survival 52.73 ± 4.08 months) as compared with those having high TUBB3 (average survival 42.56 ± 3.19 months). miR-200c also targets TrkB, a mediator of resistance to anoikis. We show that restoration of miR-200c to ovarian cancer cells results in increased anoikis sensitivity and reduced adherence to biologic substrates in vitro. Because both chemo- and anoikis-resistance are critical steps in the progression of ovarian cancer, we sought to determine how restoration of miR-200c affects tumor burden and chemosensitivity in an in vivo preclinical model of ovarian cancer. Restoration of miR-200c in an intraperitoneal xenograft model of human ovarian cancer results in decreased tumor formation and tumor burden. Furthermore, even in established tumors, restoration of miR-200c, alone or in combination with paclitaxel, results in significantly decreased tumor burden. Our study suggests that restoration of miR-200c immediately before cytotoxic chemotherapy may allow for a better response or lower effective dose. Mol Cancer Ther; 11(12); 2556–65. ©2012 AACR.

Introduction

Epithelial ovarian cancer (EOC), which accounts for 90% of ovarian cancer, is a heterogeneous disease divided into histologic subtypes: serous, mucinous, endometrioid, clear cell, Brenner, and undifferentiated carcinomas. The serous type represents 75% to 80% of EOCs and accounts for the majority of deaths from gynecologic malignancies (1–4). Even with optimal treatment consisting of surgical cytoreduction (debulking) followed by platinum- and taxane-based chemotherapy, the 5-year survival for women with advanced stage disease is only 46% at best (5). Currently there is no molecularly targeted therapy for ovarian cancer, and more than 50% of women with tumor that respond to initial treatment relapse within 18 to 24 months (5). In ovarian cancer, metastasis primarily occurs by a mechanism, termed direct seeding, which involves shedding of tumor cells from the primary site into the peritoneal cavity. For this journey, ovarian cancer cells must acquire resistance to anoikis (apoptosis initiated upon loss of attachment to native extracellular matrix) to allow survival in suspension in ascitic fluid before attaching to the peritoneum. A targeted therapy that could reduce tumor burden by simultaneously affecting multiple factors critical for ovarian cancer progression, such as anoikis resistance, chemoresistance, and attachment to sites in the peritoneal cavity, would be a revolutionary breakthrough for this aggressive gynecologic malignancy.

A recent study has shown that miRNA-200c (miR-200c) targets ZEB1 and ZEB2 in ovarian cancer cells and thereby restores E-cadherin and reduces motility (6). Subsequently, we identified class III β-tubulin gene (TUBB3) as an additional direct miR-200c target and showed that decreasing TUBB3 was the molecular mechanism whereby restoration of miR-200c increases sensitivity to paclitaxel in endometrial and ovarian cancer cells (7, 8). TUBB3 is not expressed in normal epithelial cells, but is often overexpressed in clinical specimens and cell lines of taxane-resistant carcinomas (9–12) including ovarian cancer (13, 14). Indeed, TUBB3 expression is considered to be...
one of the main mechanisms of resistance to taxanes, in general, in many types of carcinomas (9) and in ovarian cancer (13–16). The mechanism by which overexpression of TUBB3 is thought to result in resistance to taxanes, is by enhancement of the dynamic instability of microtubules, thereby counteracting the activity of microtubule-targeting agents. A recent study suggests additional novel mechanisms by which TUBB3 contributes to drug resistance (17).

Another important direct target of miR-200c that we recently identified is NTRK2, the gene encoding TrkB (18), a tyrosine kinase receptor normally expressed in neurons but coopted by various types of cancer including ovarian cancer to achieve anoikis resistance (19–23). Because chemoresistance is thought to result in resistance to taxanes, we sought to determine how restoration of miR-200c would affect ovarian cancer tumor burden and chemosensitivity in vivo. Here, we show that restoration of miR-200c to ovarian cancer cell lines enhances anoikis sensitivity, decreases attachment to biologic substrates, decreases tumor burden, and enhances chemosensitivity in vitro and in vivo.

Materials and Methods

Cell lines and tissue culture

Ovarian cell lines, HEY, SKOV3, OVCA 420, OV 1847, OVCA 433, were grown in RPMI with 10% FBS, l-glutamine and penicillin/streptomycin. Cell lines were obtained from Monique A. Spillman (University of Colorado Anschutz Medical Campus, Aurora, Colorado), who in collaboration with the University of Colorado DNA Sequencing and Analysis Core (Aurora, CO) verified the identity by DNA fingerprinting (24). All cells were used within 20 passages from receipt and less than 6 months from authentication.

Generation of stable cell lines

Stable overexpression of miR-200c was obtained with lentiviral vectors expressing miR-200c precursor sequence (pMirRNA, System biosciences).

Generation of inducible system for miR-200c expression

Hey cells were transduced with GFP-TGL-luciferase (pGL-luc retroviral vector) and selected by GFP expression. HeyTGL cells were transduced with inducible lentiviral vector pTRIPz-RFP encoding or not the precursor sequence for miR-200c (p-TRIPz-EV or pTRIPz-200c). Stable expression was selected using puromycin, and multiple clones were tested to identify those with low/absent background expression of miR-200c and at least 500-fold expression miR-200c in the presence of doxycycline (Dox) inducer.

Western blot analysis and protein expression quantification

Total cell lysates were prepared and analyzed by Western blot analysis exactly as described elsewhere (12). Primary antibodies used for Western blot analysis included mouse-monoclonal anti-TUBB3 (Sigma-Aldrich, clone B-5-1-2, 1:30,000); rabbit polyclonal anti-ZEB1 from Dr. Doug Darling (University of Louisville, Louisville, KY; 1:1,500 dilution). Secondary antibodies were Alexa-fluor 680 conjugated affinity purified anti-rabbit or anti-mouse immunoglobulin G (IgG; Invitrogen) detected using an Odyssey Infrared Imaging System (Licor Biosciences). Bands at the expected molecular weights were cropped for presentation purposes and independent blots are shown surrounded by black boxes. Protein levels were measured by obtaining the integrated intensity of the target band relative to the integrated intensity of a loading control (i.e., α-tubulin) using the Odyssey quantification tools.

Anoikis assays

Poly-hydroxyethyl methacrylate (poly-HEMA, Sigma-Aldrich) was reconstituted in 95% ethanol to a concentration of 12 mg/mL. To prepare poly-HEMA coated plates, 0.5 mL of 12 mg/mL solution was added to each well of a 24-well plate and allowed to dry overnight in a laminar-flow tissue-culture hood. Cells were transfected with transfection reagent alone, 50 nmol/L negative control or 50 nmol/L miR-200c mimics. Twenty-four hours later, 50,000 cells were plated in poly-HEMA-coated 24-well plates in the presence of 10 μmol/L EDTA. Media was collected at 8, 24, and 48 hours, and floating cells were pelleted and lysed for apoptosis analyses. For the inducible miR-200c system, the pTRIPz-200c cells were pretreated with 1 μg/mL doxycycline for 24 hours, then plated (15,000 cells/well) in 96-well plates in either adherent or suspension conditions. After 24 hours, cells were pelleted and lysed. Apoptosis was measured using the Cell Death ELISA Kit (Roche) following manufacturer’s instructions.

MTT assay

Cells were plated (1,000 per well) and treated or not with 1 μg/mL doxycycline for 24, 48, 72, and 96 hours, and cell proliferation was measured as a function of metabolism by MTT (Sigma) assay Briefly, at the indicated times, cells were incubated with 100 μL of media containing 1 μg/mL MTT for 4 hours. Formazan crystals were dissolved in 100 μL dimethyl sulfoxide and absorbance at 490 nm was recorded.

Cell-adhesion assay

Cell adhesion to vitronectin, collagen IV, fibronectin, and basement membrane complex (BM) was conducted using the Innocyte ECM cell adhesion assay (Calbiochem, Merck KGaA) according to manufacturer’s instructions.

RNA extraction and qRT-PCR

Total RNA from cultured cell lines and tumors was isolated using Trizol. cDNA from mature miR-200c was synthesized from 50 ng of total RNA using the TaqMan
MicroRNA Reverse Transcription Kit (Applied BioSystems, Life technologies) as described by the manufacturer. Quantitative real-time PCR (qRT-PCR) was conducted using TaqMan MicroRNA hsa-miR-200c Assay (Applied Biosystems, Life technologies) and the TaqMan Universal PCR Master Mix, No Amperase UNG (Applied Biosystems, Life technologies) as described by the manufacturer. RNU6 was used for normalization. At least 3 biologic replicates and triplicate PCRs were used to calculate relative expression. The relative mRNA or miRNA levels were calculated using the comparative cycle threshold (Ct) method (ΔΔCt). Briefly, the Ct values for the normalization gene were subtracted from Ct values of the target gene to achieve the ΔCt value. The 2-ΔΔCt was calculated for each sample, and then each of the values was divided by a control sample to achieve the relative mRNA or miRNA levels (ΔΔCt).

**In vitro bioluminescent assays**

Cell-expressing luciferase reporter (HeyTGL-EV or HeyTGLmiR-200c) were plated in 6-well plates and treated for indicated times with 1 μg/mL doxycycline. Ten minutes before imaging, cells were added 150 μg/mL 1-luciferin (Xenogen #XR-1001) as substrate, and total photon flux per second was measured at 0.5, 1, 5, and 10 seconds.

**Ovarian tumor xenograft and luminescent imaging in mice**

Tumor xenografts were developed by injecting 100,000 cells diluted in 100 μL PBS into the peritoneal cavity of female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Charles-River Laboratories). miR-200c expression was activated with 2 g/L doxycycline. Ten minutes before imaging, cells were added 150 μg/mL 1-luciferin in drinking water. Tumor burden was measured using IVIS 200 Optical Imaging system (Xenogen, Caliper Life Sciences) after intraperitoneal injection of 200 μL of luciferin/PBS (150 mg/kg body weight). Images were obtained 10 minutes after luciferin injection. Living Image 2.60.1 (Caliper Life Sciences) software was used for quantitative analysis at 0.5-, 1-, 5-, and 10-second time points. Images from the prone and supine position were taken and total photon flux/second per each view, from a fixed region of interest covering the abdominal area, was recorded. Total tumor burden included the total flux/second at the prone and supine positions. At termination of the experiment, mice were euthanized by CO2 asphyxiation and tumors were excised. The University of Colorado Institutional Animal Care and Use Committee (Aurora, CO) approved these experiments.

**Immunohistochemistry for TUBB3 in clinical samples of serous ovarian carcinomas and correlation with clinical outcome**

Formalin-fixed, paraffin-embedded slides obtained from 129 surgical specimens from MD Anderson Cancer Center (Houston, TX) described previously (25) were stained for TUBB3 protein by immunohistochemistry. Tissue was then stained with neuronal class IIIβ-tubulin polyclonal antibody (PRB-435P, Covance). A positive control tissue was used for normalization between staining batches.

Tumor and associated stromal TUBB3 staining were scored independently in a blinded fashion by pathologist (M.D. Post) in categories of 0, 1, 2, 3, and 4 that combined percentage of tumor cells staining and intensity of staining as follows: score 0: staining absent in tumor or weak staining in less than 10% of tumor cells (n = 20); score 1: 1% to 10% of tumor with weak or moderate staining (n = 52); score 2: more than 75% of tumor with weak staining, or less than 10% tumor with moderate to strong staining (n = 13); score 3: more than 75% tumor with moderate staining or 10% to 75% tumor with strong staining (n = 35); and score 4: more than 75% tumor with strong staining (n = 9). Stromal cells served as an internal positive control.

For survival analysis, high TUBB3 expression was defined as tumors scores of 3 or 4, moderate to high intensity staining, and greater than 10% positive tumor cells (n = 44), and low TUBB3 staining was defined as weak or moderate intensity staining in less than 10% of tumor cells (n = 52). Overall survival time was defined as the interval between initial surgical treatment and date of last follow-up or death. Association between death and TUBB3 scores was analyzed using a Cox proportional hazards model.

**Statistical analysis**

Survival analysis about the association between TUBB3 expression and death was conducted by the University of Colorado Cancer Center Biostatistics and Bioinformatics Core (Aurora, CO) using SAS/BASE and SAS/STAT software, Version 9.2 of the SAS System for Windows (SAS Institute Inc.) using a Cox proportional hazards model after confirming that the proportional hazards assumption was satisfied. The University of Colorado Department of Pathology (Aurora, CO) using GraphPad Prism 5.0 software conducted all other statistical analyses. Two-tailed Student t test (2 groups) or ANOVA followed by Tukey post hoc test (3 or more groups) were used. Results with P < 0.05 were considered statistically significant.

**Results**

**Restoration of miR-200c to ovarian cancer cells increases anoikis sensitivity and decreases adherence to components of the extracellular matrix**

To determine whether miR-200c restoration could render ovarian cancer cells sensitive to anoikis, Hey and SKOV3 cells were transiently transfected with either control or miR-200c mimics, then cultured under nonadherent conditions. Time course experiments show that miR-200c restoration significantly increases cell death in Hey and SKOV3 cells forced to grow in suspension as compared with cells transfected with a scrambled negative control mimic or mock-transfected cells under the same conditions (Fig. 1A). Relevant to attachment to
various sites in the peritoneal cavity, restoration of miR-200c to Hey cells decreases the ability to bind to components of the extracellular matrix, such as BMC, collagen IV, fibronectin, and vitronectin (Fig. 1B). Thus, miR-200c restoration results in increased anoikis sensitivity and reduced ability to adhere to biologic substrates.

An inducible system for restoration of miR-200c

Because transient miR-200c restoration has profound effects on survival of ovarian cancer cells in vitro, we sought to determine whether miR-200c restoration could affect the establishment and progression of ovarian cancer in an in vivo model. To address these questions, we engineered Hey cells to express a luciferase reporter and an inducible miR-200c lentiviral vector (HeyTGL-pTRIPz-200c) or empty vector (EV) (HeyTGL-pTRIPz-EV). Doxycycline addition to HeyTGL-TRIPz-200c cells results in a significant increase in miR-200c expression (Fig. 2A, left) and a decrease in the miR-200c targets ZEB1 (reduced to 19.5% ± 2.7%; P < 0.001) and TUBB3 (reduced to 65.9% ± 6.6%; P < 0.05; Fig. 2A, right). Doxycycline does not affect miR-200c, ZEB, or TUBB3 protein levels in the pTRIPz-EV control (data not shown). Doxycycline significantly increases cell death in HeyTGL-TRipz-200c cells growing in suspension but not in attached cells (Fig. 2B), confirming that miR-200c activation induces increased sensitivity to anoikis. Consistent with our previous findings, doxycycline activation in the pTRIPz-200c containing cells results in increased sensitivity to paclitaxel within the first 72 hours as compared with doxycycline treated TRIPz-EV, as shown by a shift in the IC₅₀ from 2.1 nmol/L (EV) to 1.4 nmol/L (Tripz-200c; Fig. 2C, left). In addition, MTT assays show that long-term doxycycline exposure (5 days) decreased cell survival in the pTRIPz-200c cells but not the EV control (Fig. 2C, right). Taken together, these data suggest that miR-200c restoration results in an increase in chemosensitivity even though cell proliferation is decreased. Because we engineered these cells to express a luciferase reporter, we determined whether the miR-200c–mediated decrease in cell survival was accurately reflected by luciferase activity using an in vivo imaging system (IVIS). Doxycycline exposure did not affect luciferase activity in wild-type Hey cells or HeyTGL-TRIPz-EV–expressing cells, but it significantly decreased the luciferase activity of HeyTGL-TRIPz-200c, indicating that miR-200c–mediated decrease in cell survival can be accurately monitored through the luciferase-based imaging system/IVIS (Fig. 2D). These data suggest that the HeyTGL-TRipz-200c cells constitute a reliable model for studying miR-200c restoration in vivo.

Stable miR-200c restoration reduces ovarian tumor formation and tumor burden

We show that miR-200c increases anoikis and results in decreased cell survival in vitro, thus we hypothesized that miR-200c might have a tumor suppressor effect in vivo in ovarian cancer. To address this question, we injected 100,000 HeyTGL-Trip-200c cells directly into the peritoneal cavity of NOD/SCID mice that had been fed (+Dox, n = 16) or not (−Dox, n = 16) with doxycycline-supplemented water. Mice were exposed to doxycycline 24 hours before cell injections and constantly thereafter, to ensure the fast upregulation of miR-200c upon cell injection. Because ovarian cancer spreads through the peritoneal cavity, restoration of miR-200c to ovarian cancer cells decreases adhesion to substrates and increases sensitivity to anoikis. A, ovarian cancer cells were mock-transfected or transfected with 50 nmol/L negative control (neg) or miR-200c mimic (200c) and 48 hours later plated on poly-HEMA coated plates in the presence of 10 μmol/L EDTA and harvested at times indicated for cell death ELISAs. B, Hey cells transduced with EV or miR-200c were assayed in a fluorescent adhesion assay to BMC, collagen type IV, fibronectin, and vitronectin. Mean of 3 biologic replicates; bars, SEM; †, P < 0.01 ANOVA.
cavity, we used IVIS to measure the luciferase signal in both the prone and supine positions. Total tumor burden was calculated by adding the total luciferase flux in the prone and supine position for each time point. As shown in Fig. 3, tumors in mice receiving miR-200c (+Dox) have less tumor burden as compared with control (−Dox) mice.

We selected 3 mice with similar tumor size in each group and conducted RT-PCR analyses of miR-200c levels. As shown in Supplementary Fig. S1, miR-200 levels were increased from 200- to 1,000-fold as compared with control mice. Thus, these data show that stable miR-200c restoration decreases tumor formation and tumor burden in vivo.

miR-200c restoration in established tumors delays tumor progression and increases sensitivity to paclitaxel

Because miR-200c restoration as a therapeutic in patients with ovarian cancer would likely occur as a
neoadjuvant or adjuvant treatment with current chemotherapies, we tested the effect of miR-200c restoration on established tumors alone or in combination with paclitaxel (8). Transient restoration of miR-200c using miRNA mimics results in a significant decrease in TUBB3 levels in not only the Hey cells, but 2 additional ovarian cancer lines that also inappropriately express TUBB3 (Supplementary Fig. S2).

We injected mice with HeyTGL-TRIPz-200c cells and allowed tumors to establish for 6 days. At this time, tumor size was measured and mice with equivalent tumor burden were matched and separated into 4 groups (Supplementary Fig. S3A). Mice were continuously supplemented with water containing doxycycline or not (+Dox or −Dox groups, respectively), and injected intraperitoneally with either vehicle or 10 mg/kg body weight paclitaxel (∼12 per group) at days 10, 18, and 24 as indicated. Tumors from mice that received +Dox+Vehicle (increased miR-200 expression only) showed an overall delay in tumor growth compared with mice where miR-200c expression was not activated (Fig. 4A). Mice in these groups were sacrificed at day 21, when the signal in the control group reached saturation (Fig. 4A). Tumors from mice receiving paclitaxel showed an overall reduction in tumor burden compared with untreated tumors (Fig. 4A and B) but progressed despite treatment with chemotherapy. Importantly, mice given doxycycline to induce miR-200c in combination with paclitaxel, showed a significant decrease in tumor burden by day 28 as compared with mice receiving paclitaxel alone (Fig. 4B). Because none of the mice reached complete remission, we tested whether miR-200c restoration had been successful in all mice. miR-200c expression was reduced in all groups receiving doxycycline (Supplementary Fig. S3B and S3C), indicating that miR-200c was indeed restored in these tumors.

Protein expression of TUBB3 significantly correlates with shorter survival

We carried out immunohistochemistry for TUBB3 on a cohort of 129 primary high-grade (stage III or IV) serous ovarian carcinomas from MD Anderson Cancer Center (described previously; ref. 25). Patients with tumors with a low-tumor TUBB3 score (1; weak staining in 1%–10% of tumor) had significantly prolonged survival (average survival 52.73 ± 4.08 months) as compared with the patients with tumors with high-TUBB3 score (3 or 4; moderate to strong staining in more than 10% of tumor; average survival 42.56 ± 3.19 months; Fig. 5). The effect on survival is no longer significant when tumors with the intermediate TUBB3 score of 2 are included in the analysis (data not shown).

Discussion

In breast and several other carcinomas, it is well established that expression of miR-200c and family members is lower in tumor versus normal tissue and that low miR-200c is clearly linked with tumor progression. In ovarian cancer, this relationship is not clear. Some studies have indicated high miR-200 family members in ovarian tumor versus normal samples (26–30). Other studies have shown no differences in miR-200 family expression in ovarian tumors (31, 32). However, some of these studies compared ovarian tumors with whole normal ovarian tissue as a control, which contains few surface epithelial cells relative to stromal cells. Only a few studies isolated “normal”
human-immortalized ovarian surface epithelial cells (HOSE) in culture and in most cases the HOSE cells were generated by various immortalization methods, which could potentially affect miR-200c levels. It is also not clear that ovarian surface epithelial (OSE) cells are always the appropriate control, as some ovarian cancers originate from fallopian tubes (33–36).

Additional complexity arises from the fact that in contrast to breast cancer, in ovarian cancer studies, it is most often metastatic-tumor that is harvested and profiled, not primary tumor. Ovarian cancer cells are thought to undergo epithelial–mesenchymal transition (EMT) to facilitate formation of spheroids in the ascitic fluid and survival before attachment at metastatic sites (37). The miR-200 family has been shown to facilitate this process because forcing expression of miR-200 members abrogates the capacity of cancer cells to form spheroids (38). Expression of miR-200 is higher in stage I EOC as compared with stage III EOC (39) indicating a downregulation of miR-200 in widespread metastasis. Interestingly, the miR-200 target, ZEB2, is expressed in cells isolated from effusions indicating that loss of miR-200 has allowed expression of this target (40, 41). Regardless of the difference in miR-200 between tumor and the appropriate “normal” control, 2 recent studies found that in both early (stage I) and advanced (stage III) ovarian cancers, low miR-200 expression is significantly associated with recurrence and poor overall survival (42, 43).

Figure 4. Restoration of miR-200c in established tumors delays tumor proliferation and increases sensitivity to paclitaxel. A total of 1 × 10⁵ HeyTGL-Tripz200c cells were injected intraperitoneally. Tumor burden was measured after 6 days and equivalent intraperitoneal tumor burdens were matched to 4 different groups (n = 12 per group). miR-200c was induced with doxycycline from day 9, and either vehicle or 10 mg/kg paclitaxel/kg body weight were injected intraperitoneally at indicated times (arrows). Tumor burden was calculated as fold change relative to values at day 6. A, top, tumor growth over time for all groups. Graph shows mean ± SEM. Bottom, scatter plot shows tumor burden in individual mice for all groups at day 21. Tumor size was significantly smaller in and miR-200c-expressing (+Dox + Vehicle) group compared with untreated (+Dox + Vehicle) tumors at day 21 (P < 0.05 Student t test). Tumor size was not statistically different in paclitaxel-treated groups (ns) by day 21. Right, image of mice in the supine position at day 21. B, left, image of mice in the supine position at day 28 in the paclitaxel-treated groups. Middle, tumor growth over time for paclitaxel-treated groups. Arrows indicate paclitaxel injection. Right, scatter plot of tumor sizes in the paclitaxel-treated (−Dox + Paclitaxel) and miR-200c + Paclitaxel-treated (+Dox + Paclitaxel) groups at the end of the experiment (day 28). Line, mean. P < 0.05 Student t test. Y-axis is plotted in Log₂ scale.
Following our report that restoration miR-200c increases sensitivity to taxanes in vitro, by directly targeting TUBB3 (7, 8), 2 independent studies showed that low tumoral miR-200c expression is significantly associated with high TUBB3 protein expression in advanced stage serous ovarian carcinomas (15, 16). Tumors of patients with minimal response to chemotherapy and low overall survival had lower miR-200c (and higher TUBB3 protein) as compared with those having complete response (16). Interestingly, patients with TUBB3 protein expression in greater than 25% of tumor cells in effusions from advanced-stage serous ovarian carcinomas had a mean overall survival of 32 months as compared with 53 months for patients with tumors with less than 25% of tumor cells staining and high TUBB3 staining also correlated with primary chemoresistance (15). Similarly, we observe a relationship between high TUBB3 protein and shorter survival in a cohort of advanced stage (III or IV) high-grade serous ovarian carcinomas. It may be of biologic significance that the results were more dramatic and more strongly predictive of outcome in the study examining tumor cells from effusions (15). We have previously shown that lack of miR-200c expression is associated with anoikis resistance and restoration of miR-200c restores anoikis sensitivity in breast and endometrial cancer cells (18), and here we observe the same to be true of ovarian cancer cells. This could indicate that TUBB3 protein may be even higher in malignant effusions (in which miR-200 is even lower) as compared with surgical specimens and may therefore be a more useful biomarker in malignant effusions.

It is highly likely that increases in other miR-200 targets in addition to ZEB1/2 and TUBB3 may play a role in tumor progression and relapse. In ovarian cancer, metastases primarily occur by direct seeding and ovarian cancer cells or spheroids must therefore acquire resistance to anoikis to survive in ascitic fluid before attaching to the peritoneum. Recently, we have shown that miR-200c restores anoikis sensitivity in aggressive breast and endometrial cancer cells by directly targeting TrkB (18). TrkB has been found to be expressed in ovarian cancer and implicated in anoikis resistance (22, 23, 44–46). Here, we show that restoration of miR-200c indeed enhances anoikis sensitivity in ovarian cancer cells. Importantly, our data show that restoration of miR-200c at the onset of tumor formation significantly diminished tumor burden in vivo. This finding supports the idea that increased anoikis sensitivity upon restoration of miR-200c via repression of TrkB or other additional targets, and/or decreased attachment to the peritoneum may contribute to the ability of this miRNA to decrease tumor burden in vivo. When we restored miR-200c to established tumors, the effect was significant, but less dramatic, suggesting that the ability of miR-200c to affect the solid tumor is slighter than its ability to reduce tumor burden when given at stages where it can increase anoikis, potentially resulting in less tumor attachment.

Here, we use an inducible in vivo model to directly test whether restoration of miR-200c would enhance the efficacy of paclitaxel in vivo. We find that restoration of miR-200c not only significantly decreases tumor burden, but also significantly enhances the response of established tumor to paclitaxel. Our study suggests that restoration of miR-200c immediately before treatment with paclitaxel could enhance response or allow for a lower effective dose initially. Alternatively, miR-200c given with paclitaxel as second-line therapy upon relapse might improve response. Further preclinical evaluations along these lines and optimization of nonviral delivery methods are warranted.

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

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References


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