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**TITLE:** Analisa DiFeo/Project Title: Identification of miRNA Signatures Associated with Epithelial Ovarian Cancer Chemoresistance with Further Biological and Functional Validation of Identified Key miRNAs

**PRINCIPAL INVESTIGATOR:** Analisa DiFeo

**CONTRACTING ORGANIZATION:** Case Western Reserve University
Cleveland, OH 44106

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<td>Analisa DiFeo, Christine Lee, Aditya Parikh, and Anil Belur Nagaraj</td>
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<tr>
<td></td>
<td>email: <a href="mailto:analisa.difeo@case.edu">analisa.difeo@case.edu</a></td>
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14. ABSTRACT
Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy in the United States. One major obstacle in the clinical management of the disease is the high incidence of recurrence after cytotoxic chemotherapy and the development of platinum resistance. Given the crucial importance to overcome chemotherapy resistance to platinum therapy, we hypothesize that miRNA profiling in EOC cell lines and surgical specimens with varying chemosensitivities will uncover a potential predictive “fingerprint” for individualized therapy, while further biological validation of these miRNAs signatures will allow for the development of novel therapeutic strategies to enhance chemosensitivity. Through microarray analysis of miRNAs differentially expressed in an *in vitro* model of acquired carboplatin resistance consisting of EOC cell lines sensitive to carboplatin, A2780, and its resistant variants, CP20 (moderately resistant) and CP70 (resistant), we identified a panel of miRNAs that correlate with carboplatin response. We uncovered four miRNAs (miR-23b, miR-132, miR-183, miR-181a and miR-203) that are significantly upregulated in both platinum-resistant cell lines. Additionally, we found that miR-181a was also correlated with several clinical parameters in a cohort of ovarian tumor specimens from women diagnosed with stage III, grade 3, papillary serous adenocarcinoma all treated with platinum-based chemotherapy. Furthermore, we uncovered the biological relevance of this miRNA. We found that miR-181a induced platinum-resistance through the maintenance of cancer stem cells via the regulation for TGFβ and Wnt signaling pathway. In summary, through the funding provided by the CDMRP we were able uncover several miRNAs that correlate with patient outcome and platinum resistance (Task 1), reveal the functional relevance of miR—181a (Task 2) and confirm that targeting this miRNA as a novel therapeutic option in ovarian cancer (Task 3).

15. SUBJECT TERMS
microRNA, ovarian cancer, platinum resistance

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**Introduction**

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. Platinum and taxane-based drugs are used in combination as first-line chemotherapeutics for women newly diagnosed with EOC, unfortunately approximately 20% do not respond to this treatment and have very poor outcomes due to chemoresistance, making advanced ovarian cancer particularly difficult to eradicate. Given the crucial importance to overcome chemotherapy resistance to platinum therapy, we hypothesize that miRNA profiling in EOC cell lines and surgical specimens with varying chemosensitivities will uncover a potential predictive “fingerprint” for individualized therapy, while further biological validation of these miRNAs signatures will allow for the development of novel therapeutic strategies to enhance chemosensitivity. The overarching goal of this project is to identify miRNAs involved in the development of ovarian cancer chemoresistance and ascertain their biological relevance using both *in vitro* and *in vivo* models of ovarian adenocarcinoma. To test our hypothesis, we propose to: 1) Validate the clinical relevance of the miRNA signature we identified in our *in vitro* model of chemosensitivity using a well-defined clinical cohort of ovarian cancer specimens; 2) determine the biological and functional relevance of the miRNAs that correlate with chemotherapeutic response; 3) explore whether *in vivo* targeting of miRNAs that are overexpressed in chemoresistant cancer cells can sensitize chemoresistant ovarian tumors to platinum treatment and inhibit ovarian cancer dissemination in a pre-clinical ovarian cancer mouse model. Our study, if successful, will identify key miRNAs involved in the regulation of chemoresistance in EOC and significantly contribute to the understanding of their biology and function *in vitro* and *in vivo*. Those miRNAs may also serve as predictive markers for tailored therapy. Furthermore, targeting these miRNAs using *in vivo* will provide a new paradigm for overcoming chemotherapy resistance clinically and thus improve survival in late stage disease.

**KEYWORDS:** ovarian cancer, platinum-resistance, microRNA, metastasis, cancer stem cells
ACCOMPLISHMENTS:
What were the major goals of the project?
Given the crucial importance to overcome tumor resistance to first-line agents such as carboplatin, we hypothesize that miRNA profiling in EOC cell lines and surgical specimens with varying chemosensitivities will uncover potential predictive markers for individualized therapy, while further biological validation of these miRNAs signatures will allow for the development of novel therapeutic strategies. Thus, the overarching goal of this proposal was to identify selected miRNAs involved in the development of ovarian cancer chemoresistance and ascertain their biological relevance using both in vitro and in vivo models of ovarian adenocarcinoma.

Task 1: Validate the clinical relevance of the panel of miRNA’s identified in our in vitro model of chemosensitivity using a well-defined clinical cohort of ovarian cancer specimens.

Task 2: Determine the biological and functional relevance of the miRNAs that correlate with chemotherapeutic response.

Task 3: Explore whether in vivo targeting of miR-183 which is overexpressed in chemoresistant cancer cells can sensitize chemoresistant ovarian tumors to cisplatin treatment and inhibit ovarian cancer dissemination in a pre-clinical ovarian cancer mouse model

What was accomplished under these goals?
Through miRNA array analysis of an in vitro model of acquired platinum resistance consisting of an OvCa cell line sensitive to carboplatin, A2780, and its resistant variants, CP20 (moderately resistant) and CP70 (resistant) which, we have identified a panel of miRNAs that are correlated with carboplatin response (Tables 1 and 2). Interestingly, the majority of miRNAs were upregulated in the platinum resistant variants compared to the parental sensitive cell line. Well-known miRNA clusters such as the miR-17-92 and the let7 family were some the most significantly altered miRNAs. Additionally, we both the moderately resistant and resistant cell lines had many of the same dysregulated miRNAs with only a few miRNA that were exclusively changed in each cell line alone (Figure 1).
We next sought to confirm our array findings and validate the clinical relevance of the panel of miRNA's identified using a well-defined clinical cohort of ovarian cancer specimens. Firstly, in order to confirm the miRNA array results several of the significantly upregulated miRNAs which have not been previously associated with chemotherapy response were analyzed in the panel of platinum-resistant ovarian cancer cell lines. Taqman PCR analysis confirmed that 4 (miR-203, -183, -23b and -132) of the 5 (miR-203, -183, -23b, -132 and -7) miRNAs analyzed were significantly upregulated in the platinum-resistant cells (Figure 2). In addition, rather than comparing two distinct groups (i.e., sensitive vs. resistant), we directly correlated the level of each miRNA expression with the IC50 of each cell line which is a measure of the effectiveness of platinum in inducing apoptosis. As shown in Figure 3, the expression of miR-203, -183, -23b and -132 reveal a positive linear correlation with cell resistance. Strikingly, miR-132 expression displays an almost perfect correlation with cell resistance ($R^2 > 0.98$).

Next, in order to assess the clinical relevance of these miRNA we ascertained whether there was a correlation between miRNA expression and several clinical parameters including progression-free survival and overall survival. Total RNA was extracted using recoverall total nucleic acid isolation kit (Ambion) from a well annotated clinical cohort of 53 stage III serous adenocarcinoma patient samples all treated with platinum therapy. Using the median expression value for each miRNA as a cut-point, the cohort was dichotomized into miRNA-high or miRNA-low expressing tumors. Interestingly, only miR-183 showed a trend towards significance with survival, high expression of miR-183 correlated with poor overall survival (Figure 4). Specifically, tumors with high expression of miR-183 had a median survival of 27.2 months whereas those patients who has low expression the median survival was 53.8 months ($p=0.056$, 95% CI 1.4-2.6).

Given that none of these preliminary miRNAs correlated with outcome we continued to expand our clinical cohort and examine the expression of other miRNAs that correlated with platinum-resistance (Table 1&2) Upon expansion of our clinical cohort we uncovered a novel miRNA, miR-181a, which was shown to be significantly induced in platinum-resistant cells (Table 2), correlated with patient outcome and tumor recurrence (Figure 5A & B). MicroRNA-181a expression dichotomized into miR-181a-high or miR-181a-low expressing tumors based on median expression in a
clinically annotated patient cohort of high-grade stage III primary papillary serous ovarian cancer, revealed significant differences in the median progression-free survival (PFS) (59.9 months) for patients with low miR-181a expression, compared to 7.1 months in patients with high miR-181a expression (Figure 5A, \(P=0.002\), OR 7.8; CI: 7.2-8.4). Furthermore, patients with low miR-181a levels exhibited a median overall survival (OS) of 66.6 months in contrast to a median OS of 24.9 months in patients with high miR-181a expression (Figure 5A, \(P=0.002\), OR 2.7; CI: 2.1-3.3). Additionally, given that a major contributor to the overall poor survival of patients with high-grade serous ovarian cancer is tumor recurrence, we also assessed whether miR-181a was differentially expressed in recurrent tumors compared to matched-primary tumors. The expression of miR-181a was assessed in patient-matched tumor biopsies that were taken at primary surgery (PS-O: chemotherapy naïve ovarian tumor) and at secondary surgery (SCR: after tumor has recurred after at least two lines of chemotherapy). In the SCR tumors, miR-181a expression was upregulated 2.51-fold compared to PS-O (\(P=0.0006\)) (Figure 5B).

Given the crucial importance of miRNAs in the regulation of global gene expression and cancer-relevant signaling pathways, our initial hypothesis was that a number of miRNAs play a yet unappreciated role in HGSOC development and chemotherapy resistance.

The other main goal of this proposal was to systemically validate the biological and functional consequences of dysregulated miRNAs that correlate with patient outcome in relevant HGSOC cell culture and in vivo models. Through these studies, we found that miR-181a can affect key biological processes including tumor cell survival, chemotherapy response, cellular migration and invasion, and cancer cell dissemination in vivo. Specifically, through the use of both intraperitoneal and intrabursal mouse models of ovarian cancer, we validated the functional relevance of miR-181a in ovarian cancer dissemination and progression in disease relevant in vivo models (Figure 6). Mice implanted with miR-181a expressing cells had increased overall tumor burden and a significantly higher incidence of metastatic nodules in the peritoneum (Figure 6A). Importantly, mRNA and IHC analyses of these tumors showed the maintenance of EMT in vivo (Figure 6B).
Following those studies we have also found that miR-181a overexpression also enhances the stem-like features of HGSOC cells that are not driven by the TGFβ signaling pathway (Figure 7). We found that miR-181a expressing cells have increased expression of stem cell markers (Figure 7B). In addition, tumor sphere assays which allow for the assessment of whether a single cell harbors the potential to both initiate and maintain tumors in the absence of cellular interaction and adhesion, revealed that enhanced miR-181a expression significantly increased CICs (Figure 7C).

Furthermore, we have now performed Extreme Limited Dilution Analysis [ELDA] in cells with targeted inhibition of miR-181a (miR-181a decoy) in order to assess miR-181a’s direct effects on stem cell frequency. One, five, ten or twenty cells/ were sorted and placed directly into 96 well ultra-low attachment plates. After 7 days, the numbers of wells with tumor spheres were counted and the data was analyzed by ELDA platform. We found that miR-181a inhibition significantly decreased the stem cell frequency (Figure 8A). Lastly, we have also completed in vivo limiting dilution assays (LDA) and determined that miR-181a increased the tumor initiating properties of primary cells (Figure 8B).

Given that uncovering the molecular factors that contribute to the emergence of CICs is a critical component to understanding HGSOC drug resistance and recurrence we next sought to determine the mechanism by which miR-181a was regulating the emergence of these CICs.

What opportunities for training and professional development has the project provided? Nothing to report

How were the results disseminated to communities of interest? A manuscript detailing some of these findings was published in Nature Communications and was featured in FierceDiagnostics (01/14); EurekAlert (01/14); ScienceDaily (01/14) and The ASCO Post (02/14). In addition, Dr. DiFeo (PI) has presented this data at several scientific meetings as well as at gynecological cancer survivor groups at local hospitals.

What do you plan to do during the next reporting period to accomplish the goals? Nothing to report
IMPACT

What was the impact on the development of the principal discipline(s) of the project?
A greater understanding of the molecular alterations driving ovarian cancer treatment resistance will allow for the development of rationally designed targeted molecular therapies to treat the underlying drivers of the disease. Clinically, 80% of women diagnosed with advanced ovarian cancer initially respond to the combination of taxane- and platinum-based chemotherapy; however, the majority of them relapses after one year and respond poorly to additional chemotherapy due to the development of drug resistance. Thus, circumventing chemoresistance to commonly use first-line agents requires innovative strategies that could ultimately improve the outcome of adjuvant therapy and result in prolonged disease-free interval and survival. This study which focused on the identification and functional assessment of key miRNAs that are involved in ovarian cancer chemoresistance lead several important findings that expand our knowledge on miRNA biology, uncovers a novel miRNA that could be used as a prognostic biomarker as well as a therapeutic target. The miRNA signature we uncovered may eventually help predict patients’ response to clinically available first-line agents and thus provide guidance for the selection of tailored chemotherapeutic regimens in order to decrease the incidence of recurrence. Lastly, uncovering that one of the miRNAs, miR-181a, drives chemotherapy resistance through the maintenance of cancer stem cells also strengthens the rationale to uncover compounds that inhibit this miRNA.

What was the impact on other disciplines?

- If there is nothing significant to report during this reporting period, state "Nothing to Report."
- Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS
Nothing to Report

PRODUCTS

Manuscripts:


Employment:

1) Received an Impact Score of 15 which was in the 1st percentile on an R01 application based on preliminary data obtained from the grant.

2) Promoted to a tenure-tracked Assistant Professor at the Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH in 2012

3) Received Norma C. and Albert I. Geller Designated Professor In Ovarian Cancer Research in 2013

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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<td>Contribution to Project:</td>
<td>Ms. Smith has performed work in the area of combined error-control and constrained coding.</td>
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<td>Funding Support:</td>
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<tr>
<td>Analisa DiFeo</td>
<td>Dr. DiFeo oversaw all experiments and interpreted the results. She guided the progress of the project.</td>
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<td>Peronne Joseph</td>
<td>Peronne performed all the experiments outlined in the grant proposal including the miRNA qPCR array on the key miRNAs that have been identified in the cell culture resistance model using 384-well customized qPCR array in the RNA samples obtained from the clinical cohort of patient specimens. She will also perform data analysis to correlate those miRNAs expression with patient response to cisplatin and other clinicopathological parameters. She also ascertained the biological relevance of each miRNAs. Peronne Joseph was responsible for data analysis, the preparation of scientific reports and manuscripts for publications.</td>
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<td>Anil Belur Nagaraj</td>
<td>Anil was responsible for the experimental design and data evaluation related to the lentiviral-based miRNA sponge/decoy vectors and pre-clinical mouse model experiments. He also helped with the preparation of manuscripts and presentations related to the overall project.</td>
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Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report