MicroRNA activity profile in the ovarian cancer cell line OVCAR3 identifies a proapoptotic effect of miR-23a

Introduction: Molecular profiling has revealed that many microRNAs (miRNAs) are highly expressed in ovarian carcinoma. However, it is not yet known which miRNAs are biologically active (ie, they suppress expression of a target gene) in ovarian cancer cells. Here we set out to determine the most active miRNAs in ovarian cancer cells.

Methods: We performed miRNA molecular profiling by quantitative polymerase chain reaction array, and measured miRNA activity using a library of sensor vectors for 291 different conserved miRNAs. We inhibited miR-23a activity using a lentiviral-based decoy, and measured the percentage of apoptotic cells by flow cytometry.

Results: Our miRNA activity profiling identified 54 active miRNAs in OVCAR3 cells, and found that over 150 miRNAs had no detectable activity. To study the function of an active miRNA, we selected miR-23a for further analysis. We inhibited miR-23a in OVCAR3 cells using a decoy vector, and found that there was decreased cell death compared to control (7.4%±1.4% versus 11.2%±0.5%; P<0.05) when the cells were treated with cisplatin. Moreover, the percentage of apoptotic cells was significantly lower in miR-23a inhibited cells compared to control (2.3%±0.4% versus 9.4%±2.6%; P<0.05).

Conclusion: This study identifies the active miRNAs in OVCAR3 cells, and suggests that miR-23a may help to regulate chemosensitivity of ovarian cancer cells.

Keywords: ovarian cancer, OVCAR3, microRNA profiling, miR-23a, apoptosis

Introduction

Ovarian cancer is the most lethal gynecological malignancy in the developed world, resulting in over 100,000 deaths per year.1 The standard treatment of advanced ovarian cancer includes debulking surgery followed by adjuvant platinum based chemotherapy. Response to chemotherapy is one of the most important predictors of survival of patients with ovarian cancer.2 Platinum-based compounds are considered the front-line chemotherapeutic agents for the treatment of patients with ovarian cancer.3 Unfortunately, despite an initial clinical response to platinum-based chemotherapy, the majority of patients develop platinum resistance following treatment. Therefore, understanding the molecular pathways regulating response to platinum-based therapy and methods of overcoming platinum resistance should help improve the outcomes of ovarian cancer patients.

microRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level.4 The 20–23 nucleotide mature miRNA is derived from a longer RNA precursor that is transcribed from the genome, and assembles into a ribonucleoprotein complex called the RNA-induced silencing complex. miRNAs guide RNA-induced silencing complex to bind to the 3′ untranslated region of
target messenger RNAs (mRNAs) that are complementary to the miRNA sequence. This results in translational repression and a decrease in the stability of the bound mRNA, which ultimately leads to reduced levels of gene expression.

miRNAs have been found to play a role in almost all biological processes, and accumulating evidence indicates that the dysregulation of miRNA expression is fundamental to the pathophysiology of human cancers. Numerous studies have demonstrated the important role of miRNAs as regulators of tumor phenotype. miRNAs control various steps in tumor progression including apoptosis, proliferation, differentiation, chemosensitivity, and metabolism. Several studies have reported the miRNA expression profiles of ovarian cancers. For example, using a miRNA microarray, Yang et al found 36 miRNAs whose expression levels were dysregulated in ovarian tumors in comparison to normal ovarian cells. The role of miRNAs in chemoresistance has also been demonstrated in several studies. Li et al demonstrated in an ovarian cancer model that miR-128 could enhance cellular uptake of cisplatin and inhibits its efflux. Zhao et al showed that miR-136 is deregulated in platinum-resistant ovarian cancer by affecting DNA repair and apoptosis.10

Measuring miRNA expression is generally used as the first step in identifying what may be the most relevant miRNAs to cell function. However, miRNA concentrations do not necessarily correlate with miRNA function. Similar to mRNAs and proteins, miRNAs are subject to complex mechanisms of post-transcriptional regulation, which influences the miRNA’s functionality even if it does not affect the miRNA’s concentration. For example, we and others have found that high concentrations of an miRNA’s targets can saturate the miRNA, and reduce its regulatory activity. It has also been shown that an miRNA can localize to subcellular compartments, and despite being expressed in the cell, it may not be able to contact its targets. Thus, just like protein profiling is limited in its ability to predict protein activity, since many types of activating and inhibiting factors may not be readily detectable, miRNA profiling has similar limitations.

To address this issue, we have developed a novel approach, dubbed Sensor-seq, which uses a collection of 100s of sophisticated miRNA “sensor” vectors to quantitate the activity of each miRNA within a cell. The collection is used as a pool, which makes the approach simple to perform. In contrast to molecular profiling, Sensor-seq provides a biological readout of an miRNA’s regulatory potential. This can be used to identify the most functionally relevant and irrelevant miRNAs within cells, and to determine if miRNAs themselves are dysregulated. As a starting point to future studies in ovarian cancer, we set out to use Sensor-seq to identify the miRNA activity profile of the ovarian cancer model OVCAR3 cell line.

Materials and methods

Cell culture

OVCAR3 cells were grown in Roswell Park Memorial Institute medium with 20% fetal bovine serum and antibiotics (1% penicillin-streptomycin). Cells were maintained at 37°C in humidified condition with 5% CO₂. No ethics statement was required from the institutional review board for the use of this cell line.

Sensor-seq

We recently developed a library of miRNA sensors and a high-throughput methodology called Sensor-seq, for employing the sensor library. The sensor is comprised of an integrating bidirectional lentiviral vector that coordinately expresses two reporter genes as distinct transcripts (Figure 1A). One reporter encodes a mutant NGFR gene, whereas the other encodes GFP linked to five tandem repeats of a perfectly complementary target site for a specific miRNA. When cells are transduced with the vector, it stably integrates into the genome. Both reporters are expressed in cells, and can be detected by fluorescent activated cell sorting (FACS). However, if the cognate miRNA is active in the cells, it will bind to its target sites in the GFP transcript, and suppress its expression in a manner that is inversely proportional to the miRNA’s activity (ie, high GFP expression indicates low miRNA activity, and low GFP expression indicates high miRNA activity). By using GFP as the reporter, we can measure the level of suppression mediated by an miRNA using FACS. This enables quantitation at single cell resolution, and the ability to flow sort the cells. NGFR was selected as the internal control because it can easily be detected by FACS using a fluorescent conjugated antibody.

After transduction of OVCAR3 cells with the miRNA sensor library, cells were FACS sorted on a FACS Vantage sorter (BD, Franklin Lakes, NJ, USA). The sorting and gate drawing were performed as previously described. Genomic DNA was isolated from FACS sorted cells using a DNeasy Blood and Tissue Kit (Qiagen NV, Venlo, the Netherlands) as per manufacturer’s recommendations. The extracted genomic DNA was prepared for deep sequencing as previously described (Figure 1). Briefly, the region of the vector containing the miRNA target sites were polymerase chain reaction (PCR) amplified as a pool using primers outside the target sites and common
to all the sensors. Barcoded adapters were ligated to the PCR amplicons, and the corresponding libraries were subjected to deep-sequencing on the Illumina HiSeq platform (Illumina, Inc., San Diego, CA, USA). We obtained 2,000,000 reads per sample on average. The sequencing reads were mapped to the sensor library and the data were normalized by total read count. This provided a frequency for each sensor in each bin which was averaged between replicates.

miRNA PCR array
We performed real-time (RT)-PCR on OVCAR3 cells using RT² miRNA PCR array (Qiagen NV). The RT-PCR was performed as per manufacturer’s recommendations.

Vector production and titration
Vectors were produced using lentiviral constructs as previously described. Briefly, 293T-cells were used for vector production. Supernatant was collected after filtration through a 0.22 µm
Vectors were concentrated by ultracentrifugation and titers were estimated on 293T-cells by limiting dilution.

**FACS**

FACS of individual sensor vectors was performed using a BD Fortessa cell analyzer. Cells were analyzed for NGFR and GFP expression. We used either FlowJo (Tree Star, Inc., Ashland, OR, USA) or FCExpress (De Novo Software, Los Angeles, CA, USA) to perform data analysis.

**Cell death and apoptosis assays**

OVCAR3 cells were transduced with individual miRNA decoys, plated in six-well plates, and treated with 4 µM of cisplatin for 24 hours. Dead cells were detected using 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining. For apoptosis assays, the cells were evaluated using the Annexin V Apoptosis Detection Kit APC (eBioscience, Inc., San Diego, CA, USA). The percentage of dead and apoptotic cells was determined by FACS.

**Quantitative real-time PCR analysis for miR-23a**

Size fractionated total RNA from OVCAR3 cells was extracted using Qiazol reagent (Qiagen NV). For detection of miR-23a, 10 ng of size fractionated total RNA was reverse transcribed into complementary DNA using TaqMan MicroRNA Reverse Transcription Kit and the specific primers designed for miR-23a (Thermo Fisher Scientific, Waltham, MA, USA). The miR-23a level was measured by real-time PCR using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). The miRNA expression was normalized using the level of miR-16 as internal control.

**Statistical analysis**

Experiments were shown as the mean ± standard deviation. Student’s t-test (two-tailed) was used to compare groups. Statistical significance was defined as P<0.05.

**Results**

**miRNA molecular profile of OVCAR3 cells by quantitative PCR (qPCR)**

To determine the miRNA profile of OVCAR3 cells, we measured the expression of 374 miRNAs in OVCAR3 cells by qPCR. The threshold cycle (CT) values were converted to arbitrary units (AU) on a linear scale using the deltaCT method with miR-16 serving as an internal calibrator. As has been previously reported for other cell lines, the miRNA profile fit a long-tail distribution with a relatively small number of miRNAs being highly expressed and the majority of miRNAs expressed at least ten fold lower (Figure 2).²¹

**miRNA activity profile of OVCAR3 cells by Sensor-seq**

Since the expression levels of an miRNA does not always correlate with its activity we performed Sensor-seq on the OVCAR3 cells to identify the most active miRNAs. Specifically, we transduced the cells with the miRNA sensor library at a low multiplicity of infection (<1) so that each cell was transduced with only a single sensor vector, as we have previously described.¹¹ Keeping the transduction rate below...
10% ensures that only a single sensor vector is integrated per cell. In our case we achieved transduction rates of 7.5% for OVCAR3 cells. As a control, cells were also transduced with the parent bidirectional lentiviral vector, which did not contain miRNA target sites in either reporter. GFP expression was different between cells transduced with the control or with the miRNA sensor library (Figure 1B). In OVCAR3 cells transduced with the parent vector, GFP and NGFR were similarly expressed. In contrast, while the majority of cells transduced with the sensor library were NGFR + GFP\textsuperscript{high} (non-active miRNAs) there were a significant number of cells that were NGFR+, but GFP\textsuperscript{low} (active miRNAs) or GFP\textsuperscript{negative} (very active miRNAs). The suppression of GFP expression indicates these cells contain sensors for active miRNAs.

To identify the suppressed sensors, and thus the active miRNAs, we sorted the cells by FACS into three bins corresponding to GFP\textsuperscript{negative} (Bin 1), GFP\textsuperscript{low} (Bin 2), and GFP\textsuperscript{high} (Bin 3) cells (Figure 1C). DNA was extracted from each group of cells, along with the total transduced population (Bin 0). We compared each sensor's frequency between bins and within the total population, (Figure 3). A significant enrichment in Bin 1 and/or Bin 2 (>1.5-fold, \(P<0.05\)), which contained GFP\textsuperscript{negative} and GFP\textsuperscript{low} cells, indicated that the sensor was suppressed and, by inference, that the corresponding miRNA was active in these cells.

In OVCAR3 cells, and using this binning approach, 34 miRNAs were rated as very active, and 20 as active (Figure 3). We did not detect activity for 190 miRNAs (Table S1).

**miR-23a is a very active miRNA in OVCAR3**

To begin to understand the function of active miRNAs in OVCAR3 cells we selected one of the very active miRNAs, miR-23a, for further analysis. We selected miR-23a since its role and function has not been studied comprehensively in ovarian cancer. As a comparative control, we used a sensor for miR-142-3p, a hematopoietic-specific miRNA\textsuperscript{22,23} that is not expressed in OVCAR3 cells, which means reporter expression can be considered maximal. The miR-23a sensor was enriched in Bin 1 and Bin 2 by 1.85- and 1.93-fold, respectively, relative to Bin 0 (Figure 4A and B). For miR-142-3p, there was no enrichment in either Bin 1 or Bin 2 compared to Bin 0; this was consistent with the fact that miR-142-3p was not detected in OVCAR3 cells by qPCR.

To validate the Sensor-seq data we created an individual miRNA sensor for miR-23a and miR-142-3p. We transduced the OVCAR3 cells with each sensor and performed FACS analysis. As shown in Figure 4C, Sensor-seq data correlated well with individual sensors’ FACS plots for both miR-23a and miR-142-3p in OVCAR3 cells.

**Knockdown of miR-23a enhances cisplatin resistance in OVCAR3 cells**

Several studies in hepatocellular and colon cancer have now demonstrated an association between miR-23a and chemoresistance.\textsuperscript{24,25} We were therefore curious to investigate a possible relationship between miR-23a and platinum chemoresistance in OVCAR3 cells. We transduced OVCAR3 cells with an miR-23a decoy. This lentiviral-based vector integrates into the genome and stably inhibits miR-23a.\textsuperscript{11} As a control, we transduced cells with a decoy for miR-142-3p which is not expressed or active in OVCAR3 cells. The miRNA decoys also express GFP. Following transduction the percentage of GFP-positive cells was >90% indicating that the vast majority of cells were expressing the decoy (Figure 5A). The decoy transcript inhibits its cognate miRNA by saturating it and by accelerating its rate of decay.\textsuperscript{15} To assess the decoy effect, we extracted size fractionated total RNA from OVCAR3 cells transduced with miR-23a decoy and control cells (without miR-23a decoy) and measured miR-23a expression by qPCR. In cells expressing the miR-23a decoy there was a 2.5-fold reduction in miR-23a, indicating that the decoy was functioning and that miR-23a was suppressed (Figure 5B).

To determine if miR-23a has a role in chemoresistance, we treated OVCAR3 cells expressing the miR-23a decoy, or the control, with cisplatin (4 \(\mu\)M) for 24 hours. We assessed cell death by DAPI staining and FACS analysis. In the parent OVCAR3 cells or cells expressing the miR-142-3p decoy, there was essentially no difference in cells undergoing cell death in response to cisplatin (12.5\%\pm2\% and 11.2\%\pm0.5\%, respectively). By contrast, in OVCAR3 cells in which miR-23a was suppressed, only 7.4\%\pm1.4\% of the cells were DAPI-positive. The difference in the percentage of dead cells was statistically significant by Student’s \(t\)-test (\(P<0.05\)) (Figure 5C). Overall these data demonstrate that OVCAR3 cells with miR-23a decoy had 1.7- and 1.5-fold decreased number of dead cells compared to controls (OVCAR3 parent cells and OVCAR3 cells expressing miR-142-3p decoy, respectively).

To further understand the role of miR-23a, we next assessed whether apoptosis was the cause of decreased cell death in OVCAR3 cells transduced with the miR-23a decoy. After cisplatin treatment we determined the frequency of apoptosis by Annexin V staining. In untransduced OVCAR3
cells treated with cisplatin an average of 7.3%±3.1% of the cells were undergoing apoptosis (Annexin V positive). Similarly, 9.4%±2.6% of the OVCAR3 cells expressing the control decoy were apoptotic. By contrast, in miR-23a suppressed OVCAR3 cells only 2.3%±0.4% of the cells were undergoing apoptosis. The difference in apoptosis between the controls and miR-23a decoy cells was statistically significant (P<0.05) (Figure 5D). Taken together, OVCAR3 cells expressing miR-23a decoy demonstrated 3.2- and 4.1-fold decrease in amount of apoptotic cells compared to controls (OVCAR3 parent cells and OVCAR3 cells expressing miR-142-3p decoy, respectively).

Figure 3 Analysis of target suppression in OVCAR3 cells by Sensor-seq. 
Notes: (A) Sensor-seq profiles of highly active microRNAs (miRNAs) in OVCAR3 cells. High activity was defined by a significant enrichment (≥1.5-fold, P<0.05) in a sensor’s frequency in Bin 1 (GFP-negative) compared to the sensor’s overall frequency in Bin 0. Shown is the mean frequency for each sensor (n=3 biological replicates). (B) Sensor-seq profiles of moderately active miRNAs in OVCAR3 cells. Moderate activity was defined by a significant enrichment (≥1.5-fold, P<0.05) in a sensor’s frequency in Bin 2 (GFP-low) compared to the sensor’s overall frequency in Bin 0. Shown is the mean frequency for each sensor (n=3 biological replicates).
MicroRNA activity profile in the ovarian cancer cells

Discussion

In our study we demonstrated both the miRNA expression and activity profile of OVCAR3 cells. Based on activity profile we selected miR-23a for further analysis and demonstrated its proapoptotic effect.

We validated our findings of concordance between Sensor-seq and individual sensors by using miR-23a and miR-142-3p as an example. Our data demonstrate the enrichment scores and individual sensor FACS plots were concordant and, in general, support the approach. We

Figure 4 miR-23a and miR-142-3p in OVCAR3 cells.
Notes: (A) Enrichment scores. (B) miR frequencies. (C) Individual sensors in OVCAR3 cells. Error bars, standard deviation; n=3 (*P<0.05).
Andikyan et al found that 34 miRNAs are highly active and 20 miRNAs are moderately active in OVCAR3 cells. As this is the first miRNA activity profile generated for an ovarian cancer cell line, we believe these data will provide a valuable resource for future studies of miRNA function in these cells. For example, one can use the activity profile to order candidate miRNAs for further functional analysis. Interestingly, while the overall trend was clear that more highly expressed miRNAs were more likely to be active, and lower expressed or undetectable miRNAs were less likely to be active, the miRNA expression levels obtained by qPCR and miRNA activity levels obtained by Sensor-seq did not correlate for all miRNAs. For example, miR-100 and miR-195 were highly expressed in OVCAR3 cells but there was little to no evidence of suppression for either sensor. This suggests that the miRNAs were inactive within the cell. This may be due to target-mediated titration or “sponging”, as has been observed in other cell types, or another mechanism, such as miRNA nuclear localization. Overall, these results provide evidence that miRNAs are subject to post-biogenesis control in OVCAR3 cells, and suggest that miRNA expression profiling alone cannot entirely predict an miRNA’s behavior within the cell. Therefore, a combination of Sensor-seq and qPCR is necessary for identifying miRNAs that are likely to be functionally significant.

miRNA profiling provides a valuable tool to identify miRNAs that are aberrantly expressed and active in different cells including cancer cell lines. The role of several active miRNAs from our analysis have been reported previously in the context of OVCAR3 cell line. For example, miR-106a was studied by Li et al. They demonstrated that miR-106a is involved in the development of drug resistance in OVCAR3 cells. Using our activity profile in OVCAR3 future research can be conducted to investigate the role of active miRNAs in ovarian cancer.

We selected one of the very active miRNAs, miR-23a, to study its role in OVCAR3 cells. Previous studies have already highlighted a role for miR-23a in chemoresistance and apoptosis. Wang et al demonstrated that overexpression of miR-23a potentiates human hepatocellular carcinoma cell to etoposide-induced cell death. Shang et al demonstrated that miR-23a expression is negatively associated with 5-fluorouracil chemosensitivity in colon cancer cells in vitro. Based on the available literature demonstrating the effect of miR-23a on chemoresistance and apoptosis we focused our attention on those properties of miR-23a. In this study we demonstrated the effect of miR-23a on chemotherapy-induced cell death and apoptosis. Here we show that suppression of miR-23a in OVCAR3 cells inhibits apoptosis and enhances chemoresistance. Thus, our data suggest that
miR-23a plays a role as a proapoptotic agent in OVCAR3 cells and is therefore a candidate for playing the same role in ovarian tumors.

**Acknowledgments**

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**

### Supplementary material

**Table S1** microRNAs with no detectable activity in OVCAR3 cells by Sensor-seq

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