Molecular Profiling Uncovers a p53-Associated Role for MicroRNA-31 in Inhibiting the Proliferation of Serous Ovarian Carcinomas and Other Cancers

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Abstract

MicroRNAs (miRNA) regulate complex patterns of gene expression, and the relevance of altered miRNA expression to ovarian cancer remains to be elucidated. By comprehensively profiling expression of miRNAs and mRNAs in serous ovarian tumors and cell lines and normal ovarian surface epithelium, we identified hundreds of potential miRNA-mRNA targeting associations underlying cancer. Functional overexpression of miR-31, the most underexpressed miRNA in serous ovarian cancer, repressed predicted miR-31 gene targets including the cell cycle regulator E2F2. MiR31 and CDKN2A, which encode p14ARF and p16INK4A, are located at 9p21.3, a genomic region commonly deleted in ovarian and other cancers. p14ARF promotes p53 activity, and E2F2 overexpression in p53 wild-type cells normally leads via p14ARF to an induction of p53-dependent apoptosis. In a number of serous cancer cell lines with a dysfunctional p53 pathway (i.e., OVCAR8, OVA433, and SKOV3), miR-31 overexpression inhibited proliferation and induced apoptosis; however, in other lines (i.e., HEY and OVSAYO) with functional p53, miR-31 had no effect. Additionally, the osteosarcoma cell line U2OS and the prostate cancer cell line PC3 (p14ARF-deficient and p53-deficient, respectively) were also sensitive to miR-31. Furthermore, miR-31 overexpression induced a global gene expression pattern in OVCAR8 associated with better prognosis in tumors from patients with advanced stage serous ovarian cancer, potentially affecting many genes underlying disease progression. Our findings reveal that loss of miR-31 is associated with defects in the p53 pathway and functions in serous ovarian cancer and other cancers, suggesting that patients with cancers deficient in p53 activity might benefit from therapeutic delivery of miR-31. Cancer Res; 70(5); 1906–15. ©2010 AACR.

Introduction

With an estimated 15,520 deaths in 2008, ovarian cancer is the fifth leading cause of cancer-associated death for women in the United States (1). Many efforts have been made to characterize ovarian cancer at the molecular level. Clinico-pathologic features, including stage, grade, and histotype, have each been associated with distinct patterns of gene expression. For example, oncogenes, such as MYC, and tumor suppressors, such as retinoblastoma (Rb) protein and p53, are each associated with better prognosis in tumors from patients with advanced stage serous ovarian cancer, potentially affecting many genes underlying disease progression. Our findings reveal that loss of miR-31 is associated with defects in the p53 pathway and functions in serous ovarian cancer and other cancers, suggesting that patients with cancers deficient in p53 activity might benefit from therapeutic delivery of miR-31. Cancer Res; 70(5): 1906–15. ©2010 AACR.
Materials and Methods

Cell cultures. After obtaining informed consent from each study participant, primary cultures of NOSE were performed as previously described (14). The epithelial origin of cultured NOSE cells was confirmed using immunohistochemistry, and only cultures containing >90% epithelial cells were used. OVCA433, U2OS, and PC3 were kindly provided by Drs. J. Wolf, L. Donehower, and M. Ittmann, respectively. Cancer cell lines were cultured in DMEM (Invitrogen; HEY, OVCA433, and U2OS), RPMI 1640 (Invitrogen; OVCAR-8, OVCAR-5, OVCAR-3, and PC3), McCoy’s 5a modified medium (Invitrogen; SKOV3), or MCDB105/M199 (Sigma; OV-90), with 10% to 20% heat-inactivated fetal bovine serum and penicillin-streptomycin (Invitrogen).

Gene expression profiling and small RNA sequencing. Total RNA was extracted from human NOSE cultures (n = 7), serous ovarian cancer cell lines (n = 5), and serous ovarian adenocarcinomas (n = 17) using the mirVana miRNA isolation kit (ABI). RNA quality and the presence of small RNAs were inspected on a 2100 Bioanalyzer (Agilent). Gene expression profiles were generated on HumanWG-6 v3.0 BeadChips (Illumina) at Texas Children’s Cancer Center Genomics and Proteomics Core Laboratory. Expression data were quantile normalized. Array data sets have been deposited into the Gene Expression Omnibus (GSE16709). Small RNA library construction was performed using the DGE-Small RNA Sample Prep kit (Illumina; n = 4 NOSE, n = 4 cell lines, n = 8 tumors). Purified cDNA was quantified with the QuantiT-IT PicoGreen dsDNA kit (Invitrogen) and diluted to 3 pmol/L for sequencing on the Illumina 1G Genome Analyzer (University of Houston). miRNA profiles were generated essentially as described in ref. 15.

Overexpression of mir-31. OVCAR-8 cells were transfected in six-well plates (2.5 × 10⁵ per well) using 7.5 μL Lipofectamine 2000 transfection reagent (Invitrogen) and 3 μg hsa-mir-31 mimic (90 nmol/L; Dharmaco) according to manufacturer’s instructions. Control groups of cells were treated with transfection reagent alone (mock transfection) or transfected with miRIDIAN hairpin inhibitor negative control #1 or mimic negative control #2 (Dharmacon). Cells were harvested 48 h after transfection, and gene expression profiling was performed as described above (n = 3 for each inhibitor or mimic; n = 3 for each negative control inhibitor or mimic).

For functional assays including proliferation and apoptosis measurements, CMV-TurboRFP-mir31-IRES-puro (OpenBio-system) was packaged and used to infect the various cell lines, according to the manufacturer’s instructions. The DNA plasmid carrying a nontargeting sequence (OpenBio-system) was used as negative control. Forty-eight hours post-transfection, cell media containing virus were syringe filtered (0.45 μm) and added onto various cell lines with appropriated dilution in the presence of polybrene (8 μg/mL). The virus-containing media were changed with fresh cell medium 6 h postinfection. The infection efficiency was measured by examining the cells under fluorescence microscope and was determined to be >95%.

Quantitative real-time PCR. Quantitative real-time PCR (QPCR) validation of microarray data was performed on samples independent of those used in microarray experiments (n = 5 for each transfection condition). Total RNA (500 ng) was reverse transcribed in a 50-μL reaction using 250 U U superscript III reverse transcriptase and random hexamers (Invitrogen). Custom primer sequences are as follows: CEIPA forward, AAGAAGTCGGTGACCAAGACAACACG; CEBPA reverse, GCAGGCGGTCATTGTCACT; serine threonine kinase 40 (STK40) forward, CTTGGCAAGAGCCGCGAGACT; STK40 reverse, GAGGCAGAAGTTGGTGATGGTT; E2F2 forward, TAGGCACTCAAGCACCTGACT; E2F2 reverse, TTGGCAACACATCGAATCTAG; QPCR was performed on an ABI Prism 7500 Sequence Detection System using SYBR Green PCR Master Mix (ABI) in a 20-μL reaction and human β-actin (ACTB) as an endogenous control.

Molecular profile analysis. Differentially expressed genes and miRNAs were identified using t test on log-transformed data and fold change (P values were two-sided). Java TreeView (16) represented expression values as color maps. miRNAs predicted to target differentially expressed mRNAs were identified using TargetScanHuman (release 5.0; ref. 17), Pictar (18), and miRanda (September 2008; ref. 19). Retrieval of putative miRNA-mRNA pairs was facilitated by SigTerms software (20). Transcriptional targets of E2F2 were defined as the set of genes elevated according to data set GSE7655 and bound by E2F2 according to ref. 21. Gene set enrichment analysis (GSEA) was executed using public software from Broad Institute (11). To score each of the Tothill and colleagues serous ovarian tumors (GSE9891) for similarity to our miR-31 gene signature, we derived a “t score” for Tothill tumor in relation to the miR-31 signature as previously described (22); progression-free survival was capped at 5 y. The mapping of transcripts or genes between array data sets was made on the Entrez Gene identifier; where multiple human array probe sets referenced the same gene, the probe set with the highest variation represented the gene. For the GSE12040 CGH data set, the 2464 BAC clones were first collapsed into the 644 cytoband loci represented by those genes; fold

(9–13); however, functional roles for most of these aberrantly expressed miRNAs have yet to be defined.

Here, we generated comprehensive miRNA and gene expression profiles for ovarian cancer by comparing papillary serous ovarian cancers, the most common cause of ovarian cancer deaths in women, with established ovarian cancer cell lines and short-term primary cultures of normal ovarian surface epithelium (NOSE). To better understand whether and how differentially expressed miRNAs affect ovarian cancers, top candidate miRNAs were experimentally altered in cell culture systems. Our findings indicate that diminished levels of miR-31 in particular (attributed in part to genomic deletion at 9p21) are correlated with defects in the p53 pathway and play a key role in ovarian cancer as well as other cancers.

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change of >1.25 was used for defining gain or loss events within each cytoband. CGH data from The Cancer Genome Atlas (TCGA)\textsuperscript{12} were from batches 9 and 11 to 13.

**Proliferation and apoptosis assays.** Three or five days after infection, cells were washed once with PBS and trypsinized. Two thousand and five hundred miR-31 overexpressed or non-targeting control overexpressed cells were resuspended in 100 μL of culture medium and seeded onto 96-well plate. Cells were allowed to settle down for 2 h, followed by the ATP quantitation–based CellTiter-Glo cell proliferation assay or an MTS-based CellTiter 96 cell proliferation assay for counting viable cells (Promega). Promega Caspase-Glo 3/7 Assay was carried out according to the manufacturer’s instructions as a sensitive assay for apoptosis.

**Results**

**miRNA expression profiles of serous ovarian cancer and integration with mRNA and DNA profiling data uncovers miR-31 as a potential tumor suppressor.** We used deep sequencing to exhaustively identify the repertoire of ~18 to 30 nucleotide small RNAs expressed in primary human serous ovarian cancers (n = 8), cell lines established from serous ovarian cancers (i.e., SKOV3, HEY, OVCAR-5, and OVCAR-8), and short-term primary cultures of human NOSE (n = 4). Approximately 50% to 70% of the (up to millions of) small RNA sequence reads for each sample mapped to known mature miRNAs in mirBase, with a total of 369 miRNAs detected in at least one sample. We found widespread differences between both cancer cell lines versus NOSE (47 miRNAs significant with P < 0.01, chance expected from multiple testing = 4) and tumors versus NOSE (42 miRNAs with P < 0.01). The top 11 miRNAs overexpressed or underexpressed in both cancer cell lines and tumors (P < 0.01, fold change of >2, each comparison) are shown in Fig. 1A (left). We found good agreement between our set of serous ovarian cancer–associated miRNAs and the results from a recent study from Wyman and colleagues (13), which also used deep sequencing to profile miRNAs in ovarian cancer (Supplementary Data Set S1).

To help examine the potential effect of global shifts in miRNA expression on the genes expressed in ovarian cancer (by virtue of miRNA-mediated mRNA destabilization), we generated gene expression profiles of serous tumors (n = 17, eight of which were also profiled for miRNA), serous cancer cell lines (the four above plus OVCAR-3, OVCA433, and Ov-90-B), and NOSE (n = 9). The gene array platform represented 48,803 mRNA transcript probes or ~24K unique named genes. We found 796 mRNA transcript probes (719 genes) higher and 1,187 probes (919 genes) lower in both cancer cell lines and tumors versus NOSE (P < 0.01, fold change of >1.5, each comparison; Fig. 1A, right; complete list provided as Supplementary Data Set S2).

To retrieve putative miRNA-mRNA functional pairs, we integrated our gene and miRNA expression profile results using the public target prediction databases TargetScan, miRanda, and PicTar. By our definition, a miRNA-mRNA functional pair consisted of a miRNA being predicted to interact with a given mRNA, wherein the two were also anticorrelated with each other in terms of expression in cancer versus normal. Both the “miRNA-down:mRNA-up” pairs (i.e., miRNA low in cancer, mRNA high in cancer) and the “miRNA-up:mRNA-down” pairs are provided as Supplementary Data Sets S3 and S4, and the overall numbers in each category (for each algorithm or intersection of algorithms) are shown in Fig. 1B.

DNA copy number alterations in cancer have been established as having a major direct role in gene and miRNA transcription patterns (12, 23). To determine whether our gene expression results (given our basis of comparison) were consistent with this notion, we obtained a set of CGH profiles from 14 serous tumors from an independent study (24), from which we estimated which cytoband regions were gained or lost in at least 50% of the tumors. There was strong overlap between genes overexpressed in cancer and genes located in regions of copy gain in cancer (with 17% of the 576 overexpressed genes represented in the CGH data showing gains, P < 5e-5, one-sided Fisher’s exact), as well as between genes underexpressed and in regions of loss (Fig. 1C). From a plot showing the frequency of DNA copy number gains and losses (Fig. 1D, top), a number of our deregulated miRNAs were in regions of gain or loss, including miR-31 (underexpressed 35-fold, ranging from 12-fold to 460-fold, in our set of tumors and showing loss in >60% of tumors at 9p21.3), which was also found in an independent study to inhibit metastasis in breast cancer (25). We checked an additional CGH data set of 178 advanced stage serous tumors from TCGA\textsuperscript{12} and found that the two CGH probes flanking miR-31 showed clear loss in ~25% of those tumors (Fig. 1D, bottom).

**Modulation of miR-31 affects predicted gene targets including genes associated with the p53 pathway.** Our bioinformatic analyses identified hundreds of putative miRNA-mRNA interactions. Reversing the expression of candidate oncopgenic or tumor suppressor miRNAs should conceivably reverse the expression patterns for in silico predicted gene targets, including those targets aberrantly expressed in cancer. Using miRNA mimics in OVCAR-8 serous ovarian cancer cells, we overexpressed miR-31, which we had found to be both underexpressed and deleted in cancer, and therefore a candidate anticancer miRNA. We then compared gene expression profiles between miR-31–transfected cells and cells transfected with a mimic control. From these data, we constructed a list of the profiled genes ordered according to higher expression in miR-31 over control (that is, the gene most induced would be at the top of this list, and the gene most repressed would be at the bottom). We next used GSEA to capture, within this ordered list, the positions of predicted miR-31 target genes, separately considering the miRanda, PicTar, and TargetScan algorithms. By eye, it was apparent that predicted targets were, in general, repressed by miR-31 (Fig. 2A), a pattern found to be statistically significant by GSEA (which yielded negative enrichment score curves),

\textsuperscript{12} http://cancergenome.nih.gov/
regardless of the target prediction algorithm considered \((P < 0.001\) for each).

Expression differences resulting from miR-31 overexpression were widespread, with 3,922 genes (4,802 probes) differing with \(P < 0.01\) (chance expected, \(\sim 488\) probes). Of the TargetScan-predicted miR-31 gene targets repressed by miR-31 overexpression, we validated STK40, CERPA, and E2F2 by QPCR (Fig. 2B), all three of which were also overexpressed in cancer versus normal and included in our putative miRNA-mRNA pairs. In addition to the predicted targets, many other genes seemed to be modulated by miR-31 (Fig. 2A), and other genes moved in the opposite direction from most targets. Whereas any one prediction algorithm likely yields many false positives, it was also conceivable that many of the miRNA-modulated genes were indirect rather than direct targets (e.g., when a miRNA modulates a transcription factor). We were able to show this in the case of E2F2, a key regulator of cell cycle genes. We obtained a set of 214 E2F2 transcriptional targets derived using published data sets from models of Drosophila melanogaster (21, 26), in which the promoters of the targets were bound by E2F2, the targets were induced (>2-fold) by E2F2 overexpression, and human orthologues of these targets were represented in our data. GSEA showed significant antienrichment of the E2F2 targets as a group within the miR-31–overexpressing cells (Fig. 2C, \(P < 0.001\)), where only 9 of the 214 genes were direct miR-31 targets by TargetScan.
We noted a number of connections between the miR-31, the E2F pathway, and the p53 pathway (Fig. 3). The CDKN2A gene, which encodes the tumor suppressor proteins p14ARF and p16INK4a, is located along with miR-31 at human chromosome 9p21.3, a region commonly deleted in cancers (27). p14ARF sequesters MDM2, the E3 ubiquitin ligase that directs p53 for degradation and also directly inhibits E2F-dependent transcription; E2Fs upregulate p14ARF, leading indirectly to p53 accumulation (28–30). Alternatively, p16INK4a functions as a cyclin-dependent kinase inhibitor for CDK4/6/cyclin D complexes important for cell cycle progression and E2F activity through regulation of the phosphorylation status of Rb (31). Furthermore, miR-31 suppresses E2F2 (Fig. 2), and the p53-dependent apoptotic program is often triggered by elevated levels and activity of E2F1 or E2F2 (32, 33). Another miR-31 target, STK40, is a repressor of p53-mediated transcription (34). The associations drawn here between miR-31, the E2F pathway, and genes related to the p53 pathway suggests a model in which miR-31 plays an anticancer role in multiple cell types, acting alone and/or in concert with p14ARF, p16INK4a, and p53 to regulate the cell cycle and the cancer (Fig. 3).

miR-31 expression induces a gene expression signature correlated with better outcome in advanced stage serous ovarian tumors. We obtained the publicly available data set from Tothill and colleagues (35) of gene expression profiles from 243 advanced stage (II–IV) serous ovarian tumors. In these tumors, we examined the expression patterns for the genes modulated (P < 0.01) by miR-31 in vitro to determine whether the miR-31 signature is present in patients and whether its presence correlates with better clinical outcome, which would further indicate that miR-31 has potential tumor suppressive abilities. Using the 3,646 miR-31 signature genes represented in the Tothill
Cox test evaluates the association of the miR-31 signature and there are significant differences between any of the three arms. Univariate P<0.05 of the signature, tumors showing deactivation (Fig. 4A). Using this approach, we found that the antiproliferative effects of miR-31 were not unique to p53-deficient serous ovarian cancers, because both the osteosarcoma cell line U2OS (p53 WT; CDKN2A mutant; ref. 44) and the prostate cancer cell line, HEY, and a clear cell ovarian cancer cell line, OVSAYO, two cell lines with wild-type p53 and CDKN2A loci (in which, interestingly, E2F2 did not seem overexpressed; Fig. 5A), miR-31 had no effect on proliferation (Fig. 5D). Importantly, we found that the antiproliferative effects of miR-31 were not unique to p53-deficient serous ovarian cancers, because both the osteosarcoma cell line U2OS (p53 WT; CDKN2A mutant; ref. 44) and the prostate cancer cell line PC3 (p53 null; CDKN2A null; ref. 45) were also sensitive to miR-31 (Fig. 6A and B, respectively).

**Discussion**

Motivated by the hypothesis that regulatory defects play an early role in the molecular changes and progression of ovarian cancer, we profiled miRNAs and their target genes in serous epithelial ovarian cancers. For further study, we focused here on miR-31, which was underexpressed in both primary cancers and established cancer cell lines. By manipulating miR-31 in vitro, we were able to show widespread effects on gene expression, as had previously been indicated by the public target prediction databases. A number of the shown miR-31 targets (e.g., E2F2) were differentially expressed between cancer and control cells and may represent genes involved early in ovarian cancer. Other miR-31 targets were potentially significant from the standpoint of cancer progression; specifically, miR-31 had widespread effects on genes correlated with poor prognosis in late-stage serous ovarian cancers.

Based on our results, miR-31 fits many of the features of a tumor suppressor candidate in serous ovarian cancer, as it is
Figure 5. Overexpression of miR-31 inhibits cancer cell proliferation in p53 pathway–inactivated ovarian cancer cell lines. A, expression patterns of E2F2, CDKN2A (p14ARF/p16INK4A gene) and well-known p53-inducible targets [e.g., p21 (CDKN1A)] in ovarian cancer cell lines and NOSE controls. The p53 and CDKN2A gene status for these cell lines as described in the literature is indicated. B, ATP quantitation–based CellTiter-Glo assay to examine the effect of miR-31 overexpression on proliferation of OVCAR8 cells (left) and caspase-3/7 activity assay for effect of miR-31 on caspase-mediated apoptosis (right). C, MTS assay for cell lines SKOV3 and OVCA433. D, MTS assay for HEY and ATP quantitation–based CellTiter-Glo assay for OVSAYO. For each time point in parts B to D delineated by asterisk (*), differences are significant with \( P < 0.05 \) (two-sided t-test). Error bars reflect results from three independent cultures.
both deleted and underexpressed; inhibits cancer cell proliferation; and increases caspase-mediated apoptosis. Recently, miR-31 was found to be linked to alterations in E2F complexes and the E2F-RB pathway (47). The CDKN2A gene, which normally activates p53 and RB functions via p14ARF to an induction of p53-dependent apoptosis (32, 33), may regulate different processes in different cancers, dependent on the cell of origin of the cancer.

Our gene signature of miR-31 overexpression might help to reveal specific miR-31–effected genes that could contribute to the progression of the disease. For example, E2F transcription factor dimerization partner 2 was repressed by miR-31 overexpression and individually correlated with poor prognosis in the Tothill data, and E2F2, although not correlated in the Tothill data set with prognosis, was correlated with increasing grade and has been shown elsewhere to be correlated with poor prognosis in ovarian cancer in other patient cohorts (46). CEBPA, which is deregulated in various cancers, was correlated here with poor prognosis; more recently, altered expression of CEBPA in prostate cancer was linked to alterations in E2F complexes and the E2F-RB pathway (47).

Mutations in TP53 have been reported in ~10% to 20% of early ovarian cancers and up to 80% of advanced serous ovarian cancers and correlate with metastatic potential (48, 49). The CDKN2A gene, which normally activates p53 and RB functions through its encoded products p14ARF and p16INK4a, is located along with miR-31 in a region of common deletion at 9p21 (27). Furthermore, studies have established a link between the RB/E2F pathway and the p53 response, wherein deregulated overexpression of E2Fs in quiescent cells normally leads via p14ARF to an induction of p53-dependent apoptosis (32, 33). Our analysis indicates that cancer cell lines with an inactive p53 functional p53 pathway were resistant to miR-31 overexpression, further suggesting a synergistic link between miR-31 deletion and/or downregulation and p53 pathway inactivation.

Therapeutic delivery of miRNAs as a means of suppressing tumor-promoting genes has potential as a cancer treatment (50), although this approach has often been shown for a particular miRNA using a single cell line or model system. Strategies to treat ovarian cancer could similarly include delivery of miR-31. At the same time, however, our study indicates that not all cancers would be susceptible to miR-31, such as those cancers that have a functional p53 pathway (including a normal/intact CDKN2A gene) or that are not driven by E2F2. This scenario observed with miR-31 could apply to the use of miRNA therapeutics in general. In the case of established therapies that directly target specific genes, such as anti-HER2 herceptin or anti-ER tamoxifen in breast cancer, only tumors that express specific molecular markers may respond. In the future, knowledge of the specific pathways targeted by a given miRNA, as well as which gene biomarkers predict therapeutic response to that miRNA, will likely be needed to properly assess the efficacy of miRNA therapeutics in controlling cancer in at least some patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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