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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 (miRNAs) 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 cell cycle regulator *E2F2*. *MIR31* and *CDKN2A*, which encodes p14^{ARF} and p16^{INK4A}, are located at 9p21.3, a genomic region commonly deleted in ovarian and other cancers. p14^{ARF} promotes p53 activity, and *E2F2* overexpression in p53 wild-type cells normally leads via p14^{ARF} to an induction of p53-dependent apoptosis. In a number of serous cancer cell lines with a dysfunctional p53 pathway (i.e., OVCAR8, OVCA433, 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 (p14^{ARF}-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

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prognosis in tumors from patients with advanced stage serous ovarian cancer, potentially impacting 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.

Keywords

microRNA; serous ovarian carcinoma; cancer therapy; miR31; TP53

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. Clinicopathologic 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 frequently misexpressed in papillary serous ovarian cancers, the most common (~70%) ovarian cancer histotype (2). While mutations in *TP53*, *BRCA1*, and *BRCA2* are each more frequently observed in poorly differentiated, high-grade serous cancers, mutations in *KRAS* and *BRAF* are more frequently observed in relatively well-differentiated, low-grade carcinomas (3–5).

MicroRNAs (miRNAs) are recently discovered small (~22 nucleotide), non-coding RNAs that play critical roles in regulating complex patterns of gene expression. Functionally, miRNAs bind to complementary sequences in the 3' untranslated region (UTR) of target gene transcripts, leading to mRNA degradation and/or translational repression (6). Thus, miRNAs add a whole new layer of complexity by which large numbers of genes and their biological processes can be broadly regulated. Dysregulated miRNA expression has been implicated in several human cancers (7), each cancer type having unique miRNA expression patterns that likely impact genes relevant to tumor pathogenesis (8). Microarray profiling studies have revealed altered miRNA expression in epithelial ovarian cancers (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, to 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 impact 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.

Materials and Methods

Cell cultures

After obtaining informed consent from each study participant, primary cultures of normal ovarian surface epithelium (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 10–20% 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=9), serous ovarian cancer cell lines (n=7), and serous ovarian adenocarcinomas (n=17) using the *mir*-Vana 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 datasets 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 Quant-iT PicoGreen dsDNA Kit (Invitrogen) and diluted to 3 pM for sequencing on the Illumina 1G Genome Analyzer (University of Houston). MicroRNA profiles were generated essentially as described in (15).

Overexpression of miR-31

OVCAR-8 cells were transfected in 6-well plates (2.5×10^5 cells/well) using 7.5 μ l Lipofectamine 2000 Transfection Reagent (Invitrogen) and 3 μ g hsa-mir-31 mimic (90 nM) (Dharmacon), 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 hours 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^R (OpenBiosystem Cat. No. HMR4842-99855788) was packaged and used to infect the various cell lines, according to the manufacturer's instructions. The DNA plasmid carrying a non-targeting sequence (OpenBiosystem Cat. No. HMR4867) was used as negative control. Forty-eight hours post-transfection, cell media containing virus were syringe filtered (0.45 μ m) and added onto various cell lines with appropriated dilution in the presence of polybrene (8 μ g/ml). The virus-containing media were changed with fresh cell medium 6 hours post-infection. The infection efficiency was measured by examining the cells under fluorescence microscope and was determined to be >95%.

Quantitative real-time PCR (QPCR)

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 μ l reaction using 250 U Superscript III reverse transcriptase and random hexamers (Invitrogen). Custom primer sequences are as follows: *CEPBA* forward, AAGAAGTCGGTGGACAAGAACAG; *CEPBA* reverse, GCAGGCGGTCATTGTCACT; *STK40* forward, CGTGACACAGAGACCTGAAGCT; *STK40* reverse, GAGGCAGAAGTTGGTGATGGTT; *E2F2* forward, TGAGCTTCAAGCACCTGACTGA; *E2F2* reverse, TTGCCAACAGCACGGATATC. QPCR was performed on an ABI Prism 7500 Sequence Detection System using SYBR Green PCR Master Mix (ABI) in a 20 μ l 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) (17), PicTar (18), and miRanda (September 2008) (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 dataset GSE7655 and bound by E2F2 according to ref (21). GSEA was executed using public software from the Broad Institute (<http://www.broad.mit.edu/gsea/>). To score each of the Tothill *et al.* 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 years. The mapping of transcripts or genes between array datasets 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 dataset, the 2464 BAC clones were first collapsed into the 644 cytoband loci represented by those genes; fold change >1.25 was used for defining gain or loss events within each cytoband. CGH data from the TCGA (<http://cancergenome.nih.gov/>) were from batch 9, and 11–13.

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 μ l of culture medium and seeded onto 96-well plate. Cells were allowed to settle down for two hours followed by the ATP quantitation-based CellTiter-Glo cell proliferation assay (Cat. No. G7570) or an MTS-based CellTiter 96 cell proliferation assay (Cat. No. G3580) for counting viable cells (Promega, Madison, WI). Promega Caspase-Glo 3/7 Assay (Cat. No. G8090) 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 ~18–30 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 normal ovarian surface epithelium (NOSE, n=4). Approximately 50–70% 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 over-expressed or under-expressed in both cancer cell lines and tumors ($p < 0.01$, fold change >2, each comparison) are shown in Figure 1A (left panel). We found good agreement between our set of serous ovarian cancer-associated miRNAs and the results from a recent study from Wyman *et al.* (13), which also used deep sequencing to profile miRNAs in ovarian cancer (Supplementary Dataset S1).

To help examine the potential impact 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, 8 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 1187 probes (919 genes) lower in both cancer cell lines and tumors versus NOSE ($P < 0.01$, fold change >1.5, each comparison) (Figure 1A, right panel) (complete list provided as Supplementary Dataset 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, where the two were also anti-correlated 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 Datasets S3 and S4, and the overall numbers in each category (for each algorithm or intersection of algorithms) are shown in Figure 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 under-expressed and in regions of loss (Figure 1C). From a plot showing the frequency of DNA copy number gains and losses (Figure 1D, top panel), a number of our deregulated miRNAs also appeared in regions of gain or loss, including miR-31 (underexpressed 35-fold, ranging from 12- 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 dataset of 178 advanced stage serous tumors from The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov/>) and found that the two CGH probes flanking miR-31 showed clear loss in about 25% of those tumors (Figure 1D, bottom panel).

Modulation of miR-31 impacts 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 oncogenic 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 anti-cancer 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, at the bottom). We next used Gene Set Enrichment Analysis (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 (Figure 2A), a pattern found to be statistically significant by GSEA (which yielded negative Enrichment Score (ES) curves), regardless of the target prediction algorithm considered ($p < 0.001$ for each).

Expression differences resulting from miR-31 overexpression were widespread, with 3922 genes (4802 probes) differing with $p < 0.01$ (chance expected ~488 probes). Of the TargetScan-predicted miR-31 gene targets repressed by miR-31 overexpression, we validated three—*STK40* (serine threonine kinase 40), *CEBPA*, and *E2F2*—by QPCR (Figure 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 appeared to be modulated by miR-31 (Figure 2A), and other genes moved in the opposite direction from most targets. While 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 demonstrate 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 datasets 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 orthologs of these targets were represented in our data. GSEA demonstrated significant anti-enrichment of the E2F2 targets as a group within the miR-31 overexpressing cells (Figure 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 miR-31, the E2F pathway, and the p53 pathway (Figure 3). The *CDKN2A* gene, which encodes the tumor suppressor proteins p14^{ARF} and p16^{INK4a} is located along with miR31 at human chromosome 9p21.3, a region commonly deleted in cancers (27). p14^{ARF} sequesters MDM2, the E3 ubiquitin ligase that directs p53 for degradation and also directly inhibits E2F-dependent transcription; E2Fs upregulate p14^{ARF}, leading indirectly to p53 accumulation (28–30). Alternatively, p16^{INK4a} 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 (Figure 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 miR31 plays an anti-cancer role in multiple cell types, acting alone and/or in concert with p14^{ARF}, p16^{INK4a}, and p53 to regulate the cell cycle and cancer (Figure 3).

miR-31 expression induces a gene expression signature correlated with better outcome in advanced stage serous ovarian tumors

We obtained the publicly-available dataset from Tothill *et al.* (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 3646 miR-31 signature genes represented in the Tothill dataset, each Tothill serous ovarian tumor was assigned a miR-31 “t-score,” which gave a measure of how the tumor recapitulated the miR-31-induced patterns of over- and under-expression (Figure 4A). Using this approach, we found that the level of enrichment of the tumors for the miR-31 signature was informative from a prognostic standpoint in the advanced stage serous tumors (Figure 4B). Those patients with tumors clearly anti-correlated in expression patterns with the signature (t-score < 0 at significance level $P < 0.05$) had a significantly shorter median time to relapse event than did those patients with tumors either clearly correlated with the signature (t-score > 0 at $P < 0.05$) or with no strong patterns either way ($P < 0.03$, Kaplan-Meier analysis, univariate Cox $P < 0.02$ when treating the t-score as a continuous variable), suggesting that loss of miR-31 activity would stimulate tumor progression.

Forced miR-31 expression inhibits proliferation in ovarian cancer, osteosarcoma, and prostate cancer cell lines

To assay the functional consequences of miR-31 overexpression in cancer, we used lentivirus to stably overexpress miR-31 *in vitro*. Because cancers of a particular histological subtype can be quite heterogeneous at the molecular level, we tested the effect of miR-31 in multiple cancer cell lines. Given the associations described above between miR-31 and the p53 pathway (e.g., whereby miR-31 suppresses E2F2, which, when overexpressed, may trigger apoptosis via p14^{ARF}), we hypothesized that inactivation of the p53 pathway (e.g., through p53 mutation or

CDKN2A deletion) provides a growth advantage in those cancers with low miR-31 and (correspondingly) high *E2F2* levels. Using our gene expression data of the ovarian cancer cell lines and normal controls, we examined expression patterns of *E2F2*, *CDKN2A* (p16^{INK4a}/p14^{ARF}), and well-established p53-inducible transcriptional targets (e.g., p21)(Figure 5A). On the basis of both the mRNA patterns and the p53 and *CDKN2A* gene status of these cell lines as documented by the literature (36–43), our cancer cell lines could be separated into those with a nonfunctional p53 pathway and those with a functional p53 pathway.

Infection of the serous ovarian cancer line, OVCAR-8 (p53-deficient), with a lentivirus encoding miR-31 slowed proliferation and caused the cells to undergo caspase-mediated apoptosis (Figure 5B) compared to a control lentivirus expressing a non-targeted miRNA sequence. We went on to overexpress miR-31 in two other serous ovarian cancer cell lines with nonfunctional p53 pathways, SKOV3 and OVCA433, where a significant inhibition of proliferation was similarly observed (Figure 5C). However, in serous ovarian 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 appear over-expressed, Figure 5A), miR-31 had no effect on proliferation (Figures 5D). Importantly, we found the anti-proliferative effects of miR-31 were not unique to p53-deficient serous ovarian cancers, since both the osteosarcoma cell line U2OS (p53 WT; *CDKN2A* mutant (44)) and the prostate cancer cell line PC3 (p53 null; *CDKN2A* null (45)) were also sensitive to miR-31 (Figures 6A and 6B, 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 demonstrate widespread effects on gene expression, as had previously been indicated by the public target prediction databases. A number of the demonstrated 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 both deleted and underexpressed, inhibits cancer cell proliferation, and increases caspase-mediated apoptosis. Recently, miR-31 was found by Valastyan *et al.* to inhibit metastasis in breast cancer (25). With our data demonstrating anti-proliferative effects of miR-31 in ovarian cancer, osteosarcoma, and prostate cancer, our study establishes an important role for miR-31 in multiple cancer types. Interestingly, however, miR-31 was not found to affect proliferation *in vitro* of MDA-MB-231 human breast cancer cells (25), and of the six demonstrated miR-31 targets in MDA-MB-231 in the Valastyan study, only two, *M-RIP* and *RDX*, were similarly downregulated ($p < 0.01$) by miR-31 in our OVCAR-8 cells. This suggests that miR-31 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-impacted genes that could contribute to the progression of the disease. For example, E2F transcription factor dimerization partner 2 (*TFDP2*), was repressed by miR-31 overexpression and individually correlated with poor prognosis in the Tothill data, and *E2F2*, while not correlated in the Tothill dataset 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 approximately 10–20% 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 p14^{ARF} and p16^{INK4a}, 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, where deregulated overexpression of E2Fs in quiescent cells normally leads via p14^{ARF} to an induction of p53-dependent apoptosis (32,33). Our analysis indicates that cancer cell lines with an inactive p53 pathway (through mutations in *TP53* and/or *CDKN2A*) were vulnerable to miR-31 overexpression, while those with a 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), though this approach has often been demonstrated 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 which have a functional p53 pathway (including a normal/intact *CDKN2A* gene) or which 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Cho K, Shih I. Ovarian cancer. *Annu Rev Pathol* 2009;4:287–313. [PubMed: 18842102]

2. Seidman J, Horkayne-Szakaly I, Haiba M, Boice C, Kurman R, Ronnett B. The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol* 2004;23:41–4. [PubMed: 14668549]
3. Levanon K, Crum C, Drapkin R. New insights into the pathogenesis of serous ovarian cancer and its clinical impact. *J Clin Oncol* 2008;26(32):5284–93. [PubMed: 18854563]
4. Bell D. Origins and molecular pathology of ovarian cancer. *Mod Pathol* 2005;18(Suppl 2):S19–32. [PubMed: 15761464]
5. Bonome T, Lee J, Park D, et al. Expression profiling of serous low malignant potential, low-grade, and high-grade tumors of the ovary. *Cancer Res* 2005;65(22):10602–12. [PubMed: 16288054]
6. Wu L, Belasco J. Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. *Mol Cell* 2008;29(1):1–7. [PubMed: 18206964]
7. Wijnhoven B, Michael M, Watson D. MicroRNAs and cancer. *Br J Surg* 2007;94(1):23–30. [PubMed: 17205498]
8. Yu S, Chen H, Yang P, Chen J. Unique MicroRNA signature and clinical outcome of cancers. *DNA Cell Biol* 2007;26(5):283–92. [PubMed: 17504024]
9. Iorio M, Visone R, Di Leva G, et al. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007;67(18):8699–707. [PubMed: 17875710]
10. Bearfoot J, Choong D, Gorringer K, Campbell I. Genetic analysis of cancer-implicated MicroRNA in ovarian cancer. *Clin Cancer Res* 2008;14(22):7246–50. [PubMed: 19010840]
11. Nam E, Yoon H, Kim S, et al. MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* 2008;14(9):2690–5. [PubMed: 18451233]
12. Zhang L, Volinia S, Bonome T, et al. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. *Proc Natl Acad Sci U S A* 2008;105(19):7004–9. [PubMed: 18458333]
13. Wyman S, Parkin R, Mitchell P, et al. Repertoire of microRNAs in epithelial ovarian cancer as determined by next generation sequencing of small RNA cDNA libraries. *PLoS One* 2009;4(4):e5311. [PubMed: 19390579]
14. Kruk PA, Maines-Bandiera SL, Auersperg N. A simplified method to culture human ovarian surface epithelium. *Lab Invest* 1990;63(1):132–6. [PubMed: 2374399]
15. Creighton C, Reid J, Gunaratne P. Expression profiling of microRNAs by deep sequencing. *Brief Bioinform*. 2009 E-pub Mar 30.
16. Saldanha AJ. Java Treeview--extensible visualization of microarray data. *Bioinformatics* 2004;20:3246–8. [PubMed: 15180930]
17. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009;19(1):92–105. [PubMed: 18955434]
18. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. *Nat Genet* 2005;37(5):495–500. [PubMed: 15806104]
19. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res* 2008;36(Database issue):D149–53. [PubMed: 18158296]
20. Creighton CJ, Nagaraja AK, Hanash SM, Matzuk MM, Gunaratne PH. A bioinformatics tool for linking gene expression profiling results with public databases of microRNA target predictions. *RNA* 2008;14(11):2290–6. [PubMed: 18812437]
21. Georlette D, Ahn S, MacAlpine D, et al. Genomic profiling and expression studies reveal both positive and negative activities for the Drosophila Myb MuvB/dREAM complex in proliferating cells. *Genes Dev* 2007;21(22):2880–96. [PubMed: 17978103]
22. Creighton C, Casa A, Lazard Z, et al. Insulin-like growth factor I (IGF-I) activates gene transcription programs strongly associated with poor breast cancer prognosis. *J Clin Oncol* 2008;26(25):4078–85. [PubMed: 18757322]
23. Pollack J, Sørli T, Perou C, et al. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc Natl Acad Sci U S A* 2002;99(20):12963–8. [PubMed: 12297621]

24. Nowee M, Snijders A, Rockx D, et al. DNA profiling of primary serous ovarian and fallopian tube carcinomas with array comparative genomic hybridization and multiplex ligation-dependent probe amplification. *J Pathol* 2007;213(1):46–55. [PubMed: 17668415]
25. Valastyan S, Reinhardt F, Benaich N, et al. A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell* 2009;137(6):1032–46. [PubMed: 19524507]
26. Cayirlioglu P, Ward W, Silver Key S, Duronio R. Transcriptional repressor functions of Drosophila E2F1 and E2F2 cooperate to inhibit genomic DNA synthesis in ovarian follicle cells. *Mol Cell Biol* 2003;23(6):2123–34. [PubMed: 12612083]
27. Sasaki S, Kitagawa Y, Sekido Y, et al. Molecular processes of chromosome 9p21 deletions in human cancers. *Oncogene* 2003;22(24):3792–8. [PubMed: 12802286]
28. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997;387(6630):296–9. [PubMed: 9153395]
29. Zhang Y, Xiong Y. Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. *Mol Cell* 1999;3(5):579–91. [PubMed: 10360174]
30. Mason S, Loughran O, La Thangue N. p14(ARF) regulates E2F activity. *Oncogene* 2002;21(27):4220–30. [PubMed: 12082609]
31. Cánepa E, Scassa M, Ceruti J, et al. INK4 proteins, a family of mammalian CDK inhibitors with novel biological functions. *IUBMB Life* 2007;59(7):419–26. [PubMed: 17654117]
32. Weinberg, R. p53 and apoptosis: master guardian and executioner. In: Weinberg, R., editor. *The biology of cancer*. Garland Science, Taylor & Francis Group, LLC; New York: 2007. p. 307-56.
33. Sherr C, McCormick F. The RB and p53 pathways in cancer. *Cancer Cell* 2002;2(2):103–12. [PubMed: 12204530]
34. Huang J, Teng L, Liu T, et al. Identification of a novel serine/threonine kinase that inhibits TNF-induced NF-kappaB activation and p53-induced transcription. *Biochem Biophys Res Commun* 2003;309(4):774–8. [PubMed: 13679039]
35. Tothill R, Tinker A, George J, et al. Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin Cancer Res* 2008;14(16):5198–208. [PubMed: 18698038]
36. Wolf J, Mills G, Bazzet L, Bast RJ, Roth J, Gershenson D. Adenovirus-mediated p53 growth inhibition of ovarian cancer cells is independent of endogenous p53 status. *Gynecol Oncol* 1999;75(2):261–6. [PubMed: 10525383]
37. Samouelian V, Maugard C, Jolicoeur M, et al. Chemosensitivity and radiosensitivity profiles of four new human epithelial ovarian cancer cell lines exhibiting genetic alterations in BRCA2, TGFb-RII, KRAS2, TP53 and/or CDKN2A. *Cancer Chemother Pharmacol* 2004;54:497–504. [PubMed: 15258697]
38. Itamochi H, Kigawa J, Kanamori Y, et al. Adenovirus type 5 E1A gene therapy for ovarian clear cell carcinoma: a potential treatment strategy. *Mol Cancer Ther* 2007;6(1):227–35. [PubMed: 17218636]
39. Havrilesky L, Alvarez A, Whitaker R, Marks J, Berchuck A. Loss of expression of the p16 tumor suppressor gene is more frequent in advanced ovarian cancers lacking p53 Mutations. *Gynecol Oncol* 2001;83:491–500. [PubMed: 11733961]
40. Kubo A, Nakagawa K, Varma R, et al. The p16 status of tumor cell lines identifies small molecule inhibitors specific for cyclin-dependent kinase 4. *Clin Cancer Res* 1999;5:4279–86. [PubMed: 10632371]
41. Mitsuchi Y, Johnson S, Selvakumaran M, Williams S, Hamilton T, Testa J. The phosphatidylinositol 3-kinase/AKT signal transduction pathway plays a critical role in the expression of p21/WAF1/CIP1/SDI1 induced by cisplatin and paclitaxel. *Cancer Res* 2000;60:5390–4. [PubMed: 11034077]
42. Yaginuma Y, Westphal H. Abnormal structure and expression of the p53 gene in human ovarian carcinoma cell lines. *Cancer Res* 1992;52:4196–9. [PubMed: 1638534]
43. Plasencia C, Dayam R, Wang Q, et al. Discovery and preclinical evaluation of a novel class of small-molecule compounds in hormone-dependent and -independent cancer cell lines. *Mol Cancer Ther* 2005;4(7):1105–13. [PubMed: 16020668]
44. Grossel M, Baker G, Hinds P. cdk6 can shorten G(1) phase dependent upon the N-terminal INK4 interaction domain. *J Biol Chem* 1999;274(42):29960–7. [PubMed: 10514479]
45. Lanzi C, Cassinelli G, Cuccuru G, et al. Cell cycle checkpoint efficiency and cellular response to paclitaxel in prostate cancer cells. *Prostate* 2001;48(4):254–64. [PubMed: 11536305]

46. Reimer D, Sadr S, Wiedemair A, et al. Clinical relevance of E2F family members in ovarian cancer--an evaluation in a training set of 77 patients. *Clin Cancer Res* 2007;13(1):144–51. [PubMed: 17200349]
47. Yin H, Lowery M, Glass J. In prostate cancer C/EBPalpha promotes cell growth by the loss of interactions with CDK2, CDK4, and E2F and by activation of AKT. *Prostate* 2009;69(9):1001–16. [PubMed: 19347879]
48. Berchuck A, Kohler M, Marks J, Wiseman R, Boyd J, Bast RJ. The p53 tumor suppressor gene frequently is altered in gynecologic cancers. *Am J Obstet Gynecol* 1994;170(1 Pt 1):246–52. [PubMed: 8296829]
49. Havrilesky L, Darcy M, Hamdan H, et al. Prognostic significance of p53 mutation and p53 overexpression in advanced epithelial ovarian cancer: a Gynecologic Oncology Group Study. *J Clin Oncol* 2003;21(20):3814–25. [PubMed: 14551300]
50. Kota J, Chivukula R, O'Donnell K, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 2009;137(6):1005–17. [PubMed: 19524505]

Figure 1. Profiling of miRNA, mRNA, and DNA in human serous ovarian tumors and cell lines. (A) Heat map representation of miRNAs (left panel) and genes (right panel) overexpressed (yellow) and underexpressed (blue) in both cell lines and tumors compared to NOSE. Rows, miRNAs or gene transcripts; columns, profiled samples. (B) Numbers of predicted miRNA-mRNA functional pairs for each algorithm and intersection of algorithms based on anti-correlated expression in ovarian cancer. (C) Percentages of genes overexpressed or underexpressed (from part A) that were located in a cytoband regions showing consistent copy number gain (orange) or loss (blue). (D) DNA copy alterations in serous ovarian cancer. *Top panel*: Frequency plot of DNA copy number gains or losses in a panel of 14 serous tumors, where locations of

microRNAs from part A are indicated. *Bottom panel:* DNA loss or gain in regions flanking miR-31 in 178 serous ovarian tumors from the TCGA.

Figure 2.

Modulation of miR-31 impacts predicted gene targets. A) Cells were transfected with miR-31 and profiled for gene expression. Genes represented in the profile dataset were ranked by fold change (overexpression/control). GSEA evaluated enrichment within the miR-31-expressing cells for predicted miR-31 targets, as determined by the given algorithm (Miranda, green; PicTar, blue; TargetScan, red). Vertical bars along the x-axis of the GSEA plot denote the positions, within the ranked list, of genes in the given set. Negative GSEA curve denotes anti-enrichment. (B) QPCR analysis showing relative quantity of miR-31 predicted targets (each normally underexpressed in cancer) after miR-31 overexpression in OVCAR-8 cells. For each gene, “miR-31 mimic” group (with overexpression of miR-31) is lower than either of the two

control groups ($p < 0.05$, two-sided t-test, each comparison). Bars indicate standard error. (C) Anti-enrichment of E2F2 transcriptional targets within miR-31 over-expressing cells, as determined by GSEA.

Figure 3.

Model for the synergistic interactions of miR-31, p16^{INK4A}, p14^{ARF}, p53, and E2F pathways in cancer. Gray lines with arrowheads denote stimulatory pathways, while black lines that end with a flat line are inhibitory pathways.

Figure 4.

Gene expression signature of miR-31 overexpression is correlated with progression in advanced stage ovarian tumors. (A) The expression patterns of the miR-31 gene signature in a panel of 243 advanced stage human serous ovarian tumors from Tothill *et al.* Tumors are ordered by the “miR-31 signature t-score” which measures the similarity of the tumor profile with the miR-31-inducible profile. (B) Kaplan-Meier analysis comparing the differences in risk of disease relapse between tumors showing activation (red line, t-score>0 and $P<0.05$) of the miR-31 signature, tumors showing deactivation (blue line, t-score<0 and $P<0.05$) of the signature, and tumor showing intermediate patterns (yellow line). Log rank test evaluates whether there are significant differences between any of the three arms. Univariate Cox test

evaluates the association of the miR-31 signature t-score with patient outcome, treating the coefficient as a continuous variable.

Figure 5. Overexpression of miR-31 inhibits cancer cell proliferation in p53 pathway-inactivated ovarian cancer cell lines. A) Expression patterns of *E2F2*, *CDKN2A* (p14^{ARF}/p16^{INK4A} 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 panel), and Caspase 3/7 activity assay for effect of miR-31 on caspase-mediated apoptosis (right panel). 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–D delineated by asterisk (“*”), differences are significant with $P < 0.05$ (two-sided t-test). Error bars reflect results from three independent cultures.

Figure 6.

Overexpression of miR-31 inhibits cancer cell proliferation in osteosarcoma and prostate cancer cell lines. An MTS assay was used to examine the effect of miR-31 overexpression on proliferation of U2OS cells (osteosarcoma, panel A) and PC3 cells (prostate, panel B).