MicroRNA Microarray Identifies *Let-7i* as a Novel Biomarker and Therapeutic Target in Human Epithelial Ovarian Cancer

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Abstract

MicroRNAs (miRNA) are approximately 22-nucleotide noncoding RNAs that negatively regulate protein-coding gene expression in a sequence-specific manner via translational inhibition or mRNA degradation. Our recent studies showed that miRNAs exhibit genomic alterations at a high frequency and their expression is remarkably deregulated in ovarian cancer, strongly suggesting that miRNAs are involved in the initiation and progression of this disease. In the present study, we performed miRNA microarray to identify the miRNAs associated with chemotherapy response in ovarian cancer and found that *let-7i* expression was significantly reduced in chemotherapy-resistant patients ($n = 69$, $P = 0.003$). This result was further validated by stem-loop real-time reverse transcription-PCR ($n = 62$, $P = 0.015$). Both loss-of-function (by synthetic *let-7i* inhibitor) and gain-of-function (by retroviral overexpression of *let-7i*) studies showed that reduced *let-7i* expression significantly increased the resistance of ovarian and breast cancer cells to the chemotherapy drug, *cis*-platinum. Finally, using miRNA microarray, we found that decreased *let-7i* expression was significantly associated with the shorter progression-free survival of patients with late-stage ovarian cancer ($n = 72$, $P = 0.042$). This finding was further validated in the same sample set by stem-loop real-time reverse transcription-PCR ($n = 62$, $P = 0.001$) and in an independent sample set by *in situ* hybridization ($n = 53$, $P = 0.049$). Taken together, our results strongly suggest that *let-7i* might be used as a therapeutic target to modulate platinum-based chemotherapy and as a biomarker to predict chemotherapy response and survival in patients with ovarian cancer. [Cancer Res 2008;68(24):10307–14]

Introduction

Epithelial ovarian cancer (EOC) is the most frequent cause of gynecologic malignancy-related mortality in women (1). Although advances in platinum-based chemotherapy have resulted in improved survival, patients typically experience disease relapse within 2 years of initial treatment and develop platinum resistance (2). Therefore, a better understanding of the mechanisms that underlie platinum resistance, including the discovery of robust predictive biomarkers which monitor the treatment and development of combination therapy that uses platinum with resistance modulators or new molecularly targeted drugs, should allow optimized therapy, such that substantial improvements in the outlook for women with this disease can be achieved (2, 3). Nevertheless, studies in the identification of druggable targets and biomarkers for ovarian cancer have thus far mainly focused on the role of protein-coding genes, whereas our knowledge of functional noncoding genomic sequences, such as microRNAs (miRNAs), is still in its infancy. miRNAs are ~22-nucleotide noncoding RNAs, which negatively regulate gene expression in a sequence-specific manner (4–6). The potential regulatory circuitry afforded by miRNA is enormous (4). Increasing evidence indicates that miRNAs are key regulators of various fundamental biological processes (4). In EOC, we have generated evidence that miRNA exhibits high-frequency genomic alterations (7), and that its expression is remarkably deregulated (8), strongly suggesting that miRNA is involved in the initiation and progression of this disease. Indeed, recent studies have shown that miRNAs play a critical role in tumor cells by serving as either oncogenes or tumor suppressor genes (5, 6), as well as by offering resistance to cytotoxic anticancer therapy (9–11). The current rapid advances in oligonucleotide/nanoparticle therapy create realistic optimism for the establishment of miRNAs as a new and potent therapeutic target and/or chemoresistant modulator in cancer treatment.

Let-7 is among the founding and best understood miRNAs in the *Caenorhabditis elegans* genome. It times seam cell terminal differentiation, possibly by acting as a regulator of multiple genes required for cell cycle and proliferation (12–15). In other organisms such as mouse, rat, and human, the *let-7* family is composed of multiple members with overlapping or distinct functions (16). Eleven members of *let-7* have been identified in the human genome (16). Most importantly, the *let-7* family is one of the first reported tumor suppressor miRNAs in cancer, which negatively regulates the RAS and is expressed at lower levels in lung tumors than in normal lung tissue (17, 18). Reduced expression of *let-7* has also been associated with shortened postoperative survival in human cancer patients (18–21). In addition, forced expression of *let-7* family members is able to suppress cancer cell growth both *in vitro* (22–24) and *in vivo* (25, 26). Finally, increasing evidence indicates

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that the *let-7* family negatively regulates numerous well-characterized oncogenic proteins, such as RAS (17, 25, 27), HMGA2 (23, 24, 27, 28), c-Myc (29), CDC25A (22), CDK6 (22), and cyclin D2 (22). Although the *let-7* family has been generally shown to be a tumor suppressor gene, there have been contradictory reports that it can serve an oncogenic function. For example, Brueckner and colleagues reported that *let-7a-3* hypomethylation results in enhanced tumor phenotype in colon cancer (30).

In the present investigation of miRNA signatures of human EOC by microarray, we found that *let-7i* is significantly reduced in chemotherapy-resistant patients and lower *let-7i* expression is strongly associated with shorter progression-free survival of the patients. *In vitro* study using various ovarian and breast cancer cell lines further confirmed that *let-7i* is involved in the cancer cell response to *cis*-platinum. Therefore, our results strongly suggest that *let-7i* might be used as a therapeutic target to modulate platinum-based chemotherapy and as a biomarker to predict chemotherapy response and survival in ovarian cancer patients.

Materials and Methods

Patients and specimens. All frozen ovarian cancer specimens used in this study were collected at the University of Turin, Turin, Italy. Clinical characteristics were as previously defined (7, 8) and listed in Table 1. Optimal surgical debulking was ≤1 cm of residual individual tumor nodules. Front-line chemotherapy comprised platinum, platinum-cyclophosphamide, or (after 1995) platinum-paclitaxel. Complete response to therapy was defined by normalization of physical examination, abdomino-pelvic computerized tomography (CT) scan and serum CA-125. Noncomplete response included partial response (≥50% decrease in the sum of greater tumor dimensions by CT) and no response (<50% decrease or any increase in tumor). Progression-free survival was the time between completion of chemotherapy and first recurrence (if a complete response had been achieved) or progression of disease, defined as ≥50% tumor increase by CT scan or two increasing CA-125 values. All tumors were from primary sites, and were immediately snap-frozen and stored at −80°C. Tissues were obtained after patients' written consent under a general tissue collection protocol approved by the Institutional Review Board of the University of Pennsylvania and the University of Turin.

Cell lines and cell culture. Ovarian (SKOV3, 2008, OVCAR10, OVCAR3), cervical (HeLa), and breast (MCF7, MDA-MB-468) cancer cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotics (Invitrogen).

RNA isolation. Total RNA was isolated from 100 to 500 mg of frozen tissue or 1×10^6 cultured cells with TRIzol reagent (Invitrogen). The quality and quantity of the isolated RNA was analyzed using a Bioanalyzer 2100 system (Agilent).

miRNA microarray. miRNA microarray was performed as previously described (8). Briefly, 5 μg of total RNA was reverse-transcribed using biotin end-labeled random-octamer oligonucleotide primer. Hybridization of biotin-labeled complementary DNA was performed on the Ohio State University miRNA microarray chip (OSU_CCC version 3.0), which contains 1,100 miRNA probes, including 326 human miRNA genes, spotted in duplicates. Often, more than one probe exists for a given mature miRNA. Additionally, there are quadruplicate probes corresponding to most pre-miRNAs. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa 647 conjugate and scanned on an Axon 4000B microarray scanner (Axon Instruments).

Microarray analysis. The normalized microarray data were managed and analyzed by GeneSpring (Agilent), GenePattern,[10] BRB-ArrayTools version 3.6,[11] and microarray software suite 4 (TM4).[12] Java Treeview 1.0 (Stanford University School of Medicine, Stanford, CA) was used for tree visualization.

Table 1. Patient characteristics (N = 72)

Characteristic	No. (%)
Age	
20–29	1 (0.01)
30–39	3 (0.04)
40–49	10 (0.14)
50–59	23 (0.32)
60–69	20 (0.28)
70–79	14 (0.19)
>80	1 (0.01)
Stage	
III	61 (0.85)
IV	11 (0.15)
Grade	
0	1 (0.01)
1	4 (0.06)
2	12 (0.17)
3	55 (0.76)
Histologic subtypes	
Serous	41 (0.57)
Endometrial	6 (0.08)
Mucinous	7 (0.10)
Clear cell	4 (0.06)
Others	14 (0.19)
Debulking status*	
Optimal (≤1 cm)	23 (0.32)
Suboptimal (>1 cm)	48 (0.67)
Chemotherapy response †	
Complete response	42 (0.58)
Noncomplete response	27 (0.38)

*One patient not available.
† Three patients not available.

Stem-loop real-time reverse transcription-PCR (TaqMan miRNA assay). Expression of mature miRNAs was analyzed by TaqMan miRNA Assay (Applied Biosystems) under conditions defined by the supplier. Briefly, single-stranded cDNA was synthesized from 5.5 ng of total RNA in a 15 μL reaction volume using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). The reactions were first incubated at 16°C for 30 min, then at 42°C for 30 min. The reactions were inactivated by incubation at 85°C for 5 min. Each cDNA generated was amplified by quantitative PCR using sequence-specific primers from the TaqMan MicroRNA Assays Human Panel on an Applied Biosystems 7900HT sequence detection system (Applied Biosystems). The 20 μL PCR included 10 μL of 2× Universal PCR Master Mix (no AmpErase UNG), 2 μL of each 10× TaqMan MicroRNA Assay Mix and 1.5 μL of reverse transcription product. The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Retroviral transduction and stable cell line generation. The retrovirus-based human miRNA expression vector was purchased from GeneService. Retroviral vector containing human *let-7i* or control vector was transfected into the packing cell line PT67 (Clontech) using FuGene6 Transfection Reagent (Roche). The medium was changed 48 h posttransfection and the medium containing retrovirus was collected 48 h later. Human tumor cells were infected with retrovirus in the presence of 8 μg/mL of of polybrene.

Transfection of inhibitor oligos. miRIDIAN inhibitors and negative controls were purchased from Dharmacon. Cells were seeded in a 96-well or

[10] http://www.broad.mit.edu/cancer/software/genepattern/
[11] http://linus.nci.nih.gov/BRB-ArrayTools.html
[12] http://www.tm4.org/

24-well plate in antibiotic-free medium to reach a 40% to 50% confluence the next day. Twenty-four hours later, the medium was replaced prior to transfection. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) following the instructions of the manufacturer. For 24-well plates, the concentration used for inhibitors was 80 nmol/L, and for 96-well plates, the concentration used for inhibitors was 66 nmol/L. Cells were incubated in the medium containing the transfection mixture for 72 h until RNA extraction (from 24-well plate) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (in 96-well plates) was performed.

***Cis*-platinum treatment.** Cells were seeded in a 96-well plate in antibiotic-free medium. *cis*-Diamineplatinum(II) dichloride (Sigma) or mock Dulbecco's PBS alone was added into the medium at various concentrations. The MTT assay was performed 72 h post-drug addition.

MTT assay. MTT assay was performed in a 96-well plate using the Cell Proliferation Kit (I) (Roche) following the manufacturer's instructions. Four to six wells were done for each sample and experiments were repeated twice. The resulting colored solution was quantified using an Emax precision microplate reader (Molecular Devices) at 570 nm with a reference wavelength of 650 nm.

Tissue microarray. The tissue microarray was constructed as described previously (8). In brief, tumors were embedded in paraffin and 5-μm sections were stained with H&E to select representative regions for biopsies. Four core tissue biopsies were obtained from each specimen. The presence of tumor tissue on the arrayed samples was verified on H&E-stained sections. The patient material consisted of 53 primary ovarian carcinomas with serous histology only. The patients were treated at the Helsinki University Central Hospital between 2000 and 2004. Patients who became disease-free after the primary treatment (surgery and platinum-taxane–based chemotherapy) were included in the study, and disease-free survival was the time from diagnosis to relapse of the disease.

miRNA *in situ* hybridization and image analysis. *In situ* detection of miRNA expression was performed on formalin-fixed paraffin-embedded tissue microarray sections. Slides were deparaffinized in xylene series and rehydrated through an ethanol series (100% to 25%). After proteinase K digestion (30 μg/mL; Roche) for 10 min and postfixation in 4% paraformaldehyde, slides were prehybridized in hybridization solution (50% formamide, 5× SSC, 500 μg/mL yeast tRNA, 1× Denhardt's solution) for 1 h and hybridized overnight with digoxigenin-labeled miRNA-locked nucleic acid probe (Exiqon) in hybridization solution. After stringent washes (50% formamide, 2× SSC) at hybridization temperature, chromogenic detection of signals was performed using anti-digoxigenin antibody (Roche, 1:400 dilution) and PowerVision+ Poly-HRP IHC detection kit (ImmunoVision Technologies) according to the manufacturer's instructions. Occasionally, a nuclear signal was seen most likely representing nonspecific staining as it was also seen in the negative controls. Therefore, only cytoplasmic staining (mature miRNA) of the tumor cells was recorded and classified as positive or negative without knowledge of the patient outcome.

Array-based comparative genomic hybridization. BAC clones included in the "1 Mb array" platform were recently described (7). Briefly, 4,134 clones from the CalTech A/B and RPCI-11 libraries were collected from both commercial and private sources and were mapped to build 34 of the human genomes using either an STS-marker (29%), end sequences (68%), or full sequences (3%). A minimum of two replicates per clone were printed on each slide. One microgram of tumor and reference DNA was labeled with Cy3 or Cy5, respectively (Amersham) using the BioPrime random-primed labeling kit (Invitrogen). In parallel experiments, tumor DNA and reference DNA were labeled with the opposite dye to account for differences in dye incorporation and to provide additional data for analysis. A systematic protocol was used to analyze array-based comparative genomic hybridization (aCGH) data for copy number alterations. For quality control purposes, clones demonstrating an adjusted foreground-to-background intensity ratio of <0.8 in the reference channel were removed. With dye swap data merged as input, copy number breakpoints were estimated for each sample by the Circular Binary segmentation algorithm using breakpoint significance based on 10,000 permutations. Additional analyses and visualization of aCGH data were done using the CGHAnalyzer software suite described previously.

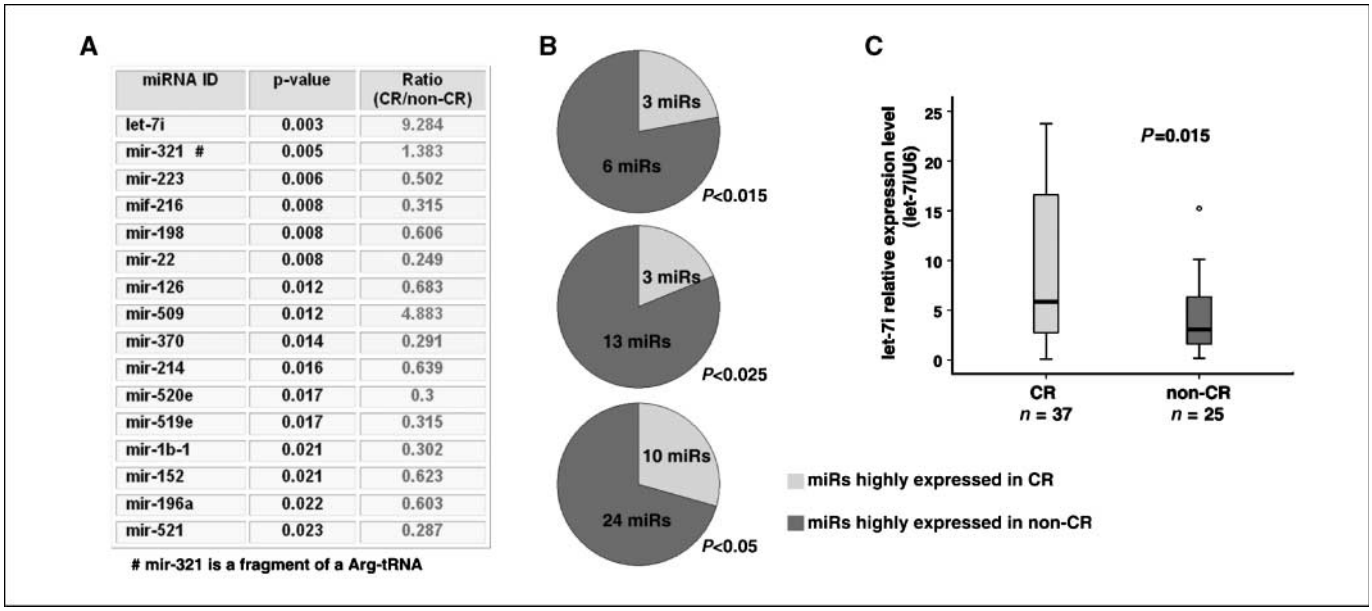
Statistical analysis. Statistical analysis was performed using the SPSS statistics software package (SPSS). All results were expressed as mean ± SD, and $P < 0.05$ was used for significance. Kaplan-Meier curves were used to estimate 5-year survival rates and were compared with the use of log rank statistics.

Results

***Let-7i* expression is significantly reduced in patients with chemotherapy-resistant EOC.** To identify miRNA expression signatures associated with resistance to chemotherapy in patients with EOC, specimens from 72 late-stage (stage III and IV) patients were initially analyzed by miRNA microarray. A total of 69 patients with well-documented chemotherapy response information were included for further biomarker identification, and all ($n = 72$) were used for survival analysis. The clinical characteristics of those patients are listed in Table 1. First, differences in miRNA expression between the complete response ($n = 42$) and noncomplete response groups ($n = 27$, including partial response and no response) were analyzed. It was found that 34 miRNAs were statistically different ($P < 0.05$) between the groups, with 24 (70.6%) miRNAs higher in the noncomplete response group and 10 miRNAs (29.4%) higher in the complete response group (Supplementary Table S1). Importantly, nine miRNAs exhibited even greater statistical significance ($P < 0.015$) and of those, six were higher in the noncomplete response group and three were higher in the complete response group (Fig. 1*A* and *B*). In particular, *let-7i*, a tumor suppressor miRNA (17, 18), was the top differential miRNA between the two groups and expressed at remarkably lower levels in the noncomplete response group (expression ratio of complete response group to noncomplete response group = 9.3, $P = 0.003$, $n = 69$; Fig. 1*A*). To further validate this finding, we examined *let-7i* expression in 62 randomly selected late-stage EOC specimens by stem-loop real-time reverse transcription-PCR. Consistent with the microarray data, *let-7i* expression was indeed significantly reduced in the noncomplete response patients (9.1 ± 1.5 relative expression unit; *let-7i*/U6; $n = 25$) as compared with their counterparts with complete response (4.3 ± 0.7 relative expression unit; *let-7i*/U6; $n = 37$, $P = 0.015$; Fig. 1*C*). In addition, this result was further confirmed in EpCAM-positive tumor cells isolated from the ascites of late-stage ovarian cancer patients (~13.9-fold higher in enriched tumor cells from the complete chemotherapy response patients compared with those from the noncomplete chemotherapy patients; $n = 8$).[13] Taken together, we found that there was a distinguishable miRNA expression signature between the chemotherapy-responsive and chemotherapy-resistant EOC patients, and expression of the tumor suppressor miRNA *let-7i* was significantly reduced in the chemotherapy-resistant EOC patients.

Decreased *let-7i* expression increases the chemotherapy resistance of EOC cells. Studies from our group and other groups have demarcated that miRNAs are globally down-regulated in human cancers including EOC (8, 31). Those down-regulated miRNAs, such as the *let-7* family (17, 18), might serve as tumor suppressor genes and their suppression can have an important effect on tumor cells, e.g., by rendering them more resistant to cytotoxic anticancer therapy (9–11). *let-7i* has been reported to be

[13] Unpublished observation.



miRNA ID	p-value	Ratio (CR/non-CR)
let-7i	0.003	9.284
mir-321 #	0.005	1.383
mir-223	0.006	0.502
mif-216	0.008	0.315
mir-198	0.008	0.606
mir-22	0.008	0.249
mir-126	0.012	0.683
mir-509	0.012	4.883
mir-370	0.014	0.291
mir-214	0.016	0.639
mir-520e	0.017	0.3
mir-519e	0.017	0.315
mir-1b-1	0.021	0.302
mir-152	0.021	0.623
mir-196a	0.022	0.603
mir-521	0.023	0.287

mir-321 is a fragment of a Arg-tRNA

Figure 1. *let-7i* expression is significantly reduced in patients with chemotherapy-resistant EOC. *A,* microarray analysis of miRNA expression between complete response (*CR*) and noncomplete response (*non-CR*) ovarian cancer patients. *B,* differentially expressed miRNAs between complete response and noncomplete response patients at various statistical significance ($P < 0.015$, $P < 0.025$, and $P < 0.05$). *C,* validation of *let-7i* expression in complete response and noncomplete response patients by real-time reverse transcription-PCR.

down-regulated in recurrent ovarian tumors compared with primary tumors (32). Therefore, to further investigate whether the above identified miRNAs are functionally involved in tumor resistance to chemotherapy, three miRNAs (*let-7i, mir-321*, and *mir-509*; Fig. 1*A*) that were significantly repressed in the chemotherapy-resistant tumors were focused on. *mir-321*, a fragment of Arg-tRNA, was excluded from our study, and both mature forms of *mir-509* (*mir-509-5p* and *mir-509-3p*) were included. A total of three mature miRNAs, *let-7i, mir-509-5p*, and *mir-509-3p* were examined in EOC cell lines (2008 and SKOV3) *in vitro*. Endogenous miRNA expression was blocked by specific antisense oligonucleotide inhibitors. The effect on miRNA expression by the inhibitor was confirmed by stem-loop real-time reverse transcription-PCR. More than 90% of the endogenous miRNA expression was blocked by the inhibitor 48 hours posttransfection (Fig. 2). It was found that knockdown of the *let-7i* expression, but not that of *mir-509-3p* or *mir-509-5p*, significantly increased cell resistance to *cis*-platinum treatment in various EOC cell lines (2008, $P = 0.004$; SKOV3, $P = 0.006$; Fig. 2*A*). A similar result was also found in short-term primary cultured ovarian tumor cells (Supplementary Fig. S1). To complement this loss-of-function study, we also stably enforced *let-7i* expression in EOC (2008 and SKOV3) and breast (MCF7) cell lines via retroviral transduction before exposing them to serial concentrations of *cis*-platinum. Overexpression of *let-7i* in each of the above cell lines was confirmed by stem-loop real-time reverse transcription-PCR (Fig. 2*C*). Consistent with the loss-of-function study, overexpression of *let-7i* significantly increased the chemotherapy response sensitivity *in vitro* (Fig. 2*C*). Taken together, down-regulated or intrinsically reduced *let-7i* expression could render EOC cells more resistant to the *cis*-platinum treatment. Therefore, *let-7i* might serve as an important chemotherapy response modulator in cancer cells.

***let-7i* DNA copy number does not exhibit genomic alteration in human cancer.** The molecular mechanism of *let-7i* down-regulation in patients with chemotherapy-resistant EOC is unclear. Our previous studies indicated that DNA copy number of miRNAs is highly altered in human cancer including EOC (7), and DNA copy number alteration significantly contributes to miRNA expression in cancer. For example, *let-7a3* deletion was found in 31.2% of EOC specimens ($n = 106$), which significantly reduced *let-7a3* expression in EOC (7). Therefore, we questioned whether DNA copy alteration of *let-7i* contributes to the reduced expression of *let-7i* in patients with chemotherapy-resistant EOC. In the 69 patients that were used for initial analysis of chemotherapy-associated miRNA markers, 30 were analyzed by aCGH (complete response patients, $n = 20$; and noncomplete response patients, $n = 10$). We first analyzed the genomic locus, Chr12_61-62 Mb, which contains the primary *let-7i* gene sequence, in these specimens. However, there was only one patient with a *let-7i* DNA copy number alteration in the chemotherapy response group (1/30, 3.3%), no patients with either deletion or application were found in the chemotherapy-resistant group. This indicates that unlike other *let-7* family members, *let-7i* does not significantly exhibit DNA copy alteration in EOC. Therefore, DNA copy number alteration might not affect *let-7i* reduced expression in patients with chemotherapy-resistant EOC. For future confirmation of this conclusion, we expanded our aCGH study to a large collection of specimens with multiple cancer types including nine different types of human solid tumors (bladder breast, colon, lung, ovarian and pancreatic cancer, sarcoma, neuroblastoma, and melanoma; $n = 1{,}315$; Fig. 3 and Supplementary Table S2; ref. 33). Consistent with the first analysis, the DNA copy number of *let-7i* was found in only extremely low frequency alterations (gained three to five copies in 5% and heterogeneously deleted in 6%, <10% alteration was usually considered as the background signal of aCGH), which was significantly lower than other members of the *let-7* family (e.g., *let-7a-3* and *let-7b* deleted in 31.2% of EOC; ref. 7). These results suggested that other unknown mechanisms reduced *let-7i* expression in the chemotherapy-resistant patients, e.g., mutation, miRNA biogenesis pathway (34), epigenetic, or transcriptional regulation.

Low *let-7i* expression is significantly associated with shorter survival of patients with EOC. It has been reported that the expression of *let-7* family is a strong prognostic marker for human cancer patients (18–21). In this study, we identified *let-7i* as an important predictor for chemotherapy resistance in patients with EOC. We further investigated whether *let-7i* could also serve as a prognostic marker in patients with EOC. To examine the correlation between *let-7i* expression and rapid recurrence of the disease, we first studied the *let-7i* expression in 72 late-stage EOC patient samples by miRNA microarray. Kaplan-Meier survival analysis indicated that low expression of *let-7i* was significantly associated with shorter progression-free survival of the patients as compared with the high *let-7i* expression group ($P = 0.042$, $n = 72$; Fig. 4*A*). We then validated this result by a more accurate mature miRNA quantitative method in the same sample set. Consistently, a similar result was also observed in the 62 randomly selected EOC patient samples analyzed by stem-loop real-time reverse transcription-PCR ($n = 62$, $P = 0.001$; Fig. 4*B*). Finally, we analyzed *let-7i* expression in an independent sample set using a completely different methodology—*in situ* hybridization. Again, we found that

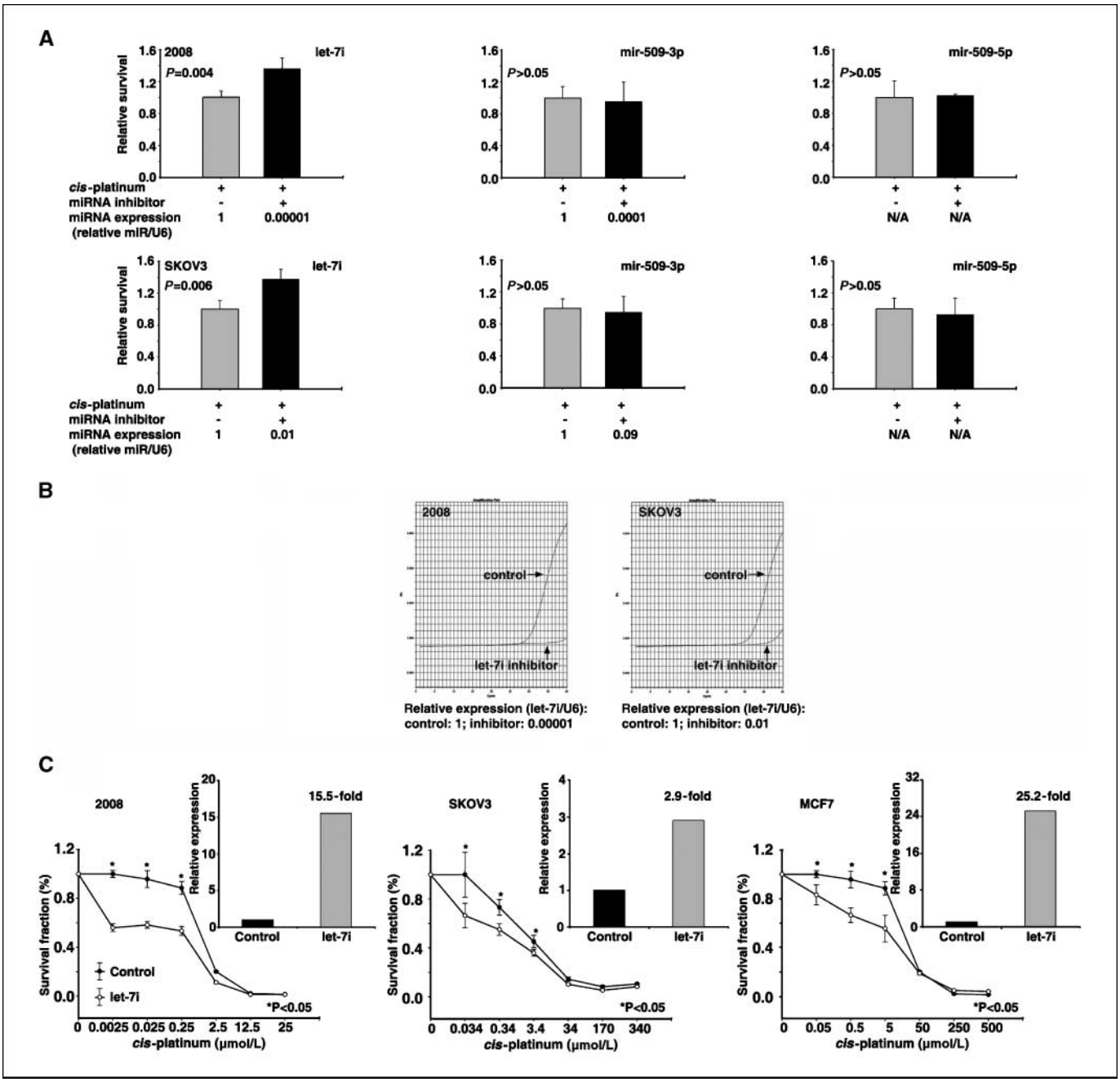


Figure 2. *let-7i* expression regulates *cis*-platinum resistance of EOC cells. *A,* inhibition of *let-7i*, but not *mir-509-3p* or *mir-509-5p*, increased resistance to *cis*-platinum treatment in 2008 and SKOV3 cells. *B,* stem-loop real-time reverse transcription-PCR showed endogenous *let-7i* was significantly blocked by *let-7i* inhibitor. *C,* overexpression of *let-7i* by retroviral infection in 2008, SKOV3, and MCF7 cells increased their sensitivity to the *cis*-platinum treatment. *Inset,* stem-loop real-time reverse transcription-PCR showed that *let-7i* was stably overexpressed in EOC cell lines by retroviral transfection.

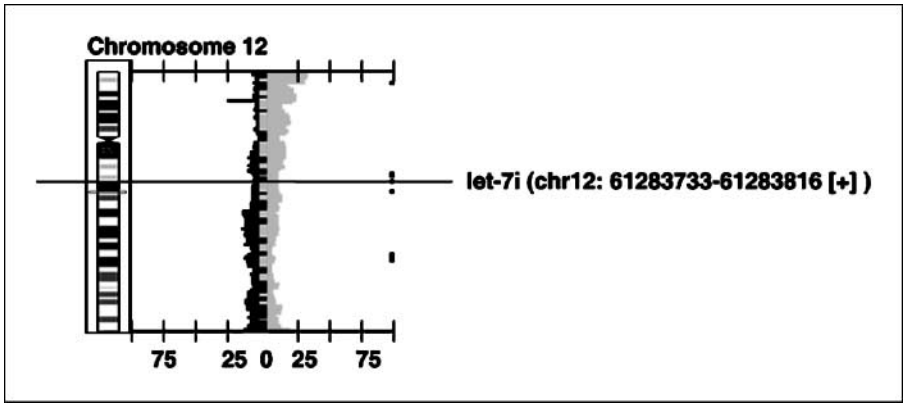


Figure 3. *let-7i* DNA copy number does not exhibit genomic alteration in human cancer. Genomic locus harboring *let-7i* did not exhibit alteration in EOC (n = 106). *Black,* deletion; *gray,* amplification.

lower *let-7i* expression was significantly associated with shorter disease-free survival in 53 samples examined by *in situ* hybridization of tissue array (n = 53, P = 0.049; Fig. 4*C*). In conclusion, the above data strongly suggests that the expression level of *let-7i* could serve as a novel prognostic and prediction biomarker for the survival of patients with EOC.

Discussion

EOC is the most lethal gynecological malignancy in western countries (1). The role of miRNAs in ovarian cancer has recently been proposed and investigated (7, 8, 20, 21, 32, 35–37), which might offer novel strategies for prevention, early detection, diagnosis, and treatment of this disease. Here, we used miRNA microarray on 69 ovarian tumor specimens to identify the miRNA signature associated with chemotherapy response in ovarian cancer. *Let-7i*, a *let-7* family member, was found to be an important miRNA, differentiating ovarian cancer patients with complete response or noncomplete response to chemotherapy. Further investigation using a variety of cultured cancer cell lines confirmed that *let-7i* is as functionally involved in the tumor cells response to *cis*-platinum. The present study may therefore provide a novel prognostic biomarker and therapeutic target for ovarian cancer.

The role of *let-7* in cancer was first shown by the Slack group when they found that the *let-7* family negatively regulates *let-60/RAS* in *C. elegans* by binding to the multiple *let-7* complementary sites in its 3′-untranslated region (17). Moreover, having found that *let-7* expression is lower in lung tumors than in normal lung tissue, whereas RAS protein is significantly higher in lung tumors, they proposed *let-7* as a tumor suppressor gene (17), which is consistent with previous clinical observation in lung cancer (18). The inhibitory function of the *let-7* family in cancer has been corroborated by a number of groups and in various types of tumors (9–11, 17, 18, 20–23, 25–29, 34, 35, 38). *Let-7* probably performs those functions by targeting various genes. First, *let-7* inhibits several well-characterized oncogenic proteins such as K-RAS (17, 25, 27), H-RAS (17, 25, 27), HMGA2 (23, 24, 27, 28), c-Myc (29), and NF2 (11). In addition, *let-7* may target multiple cell

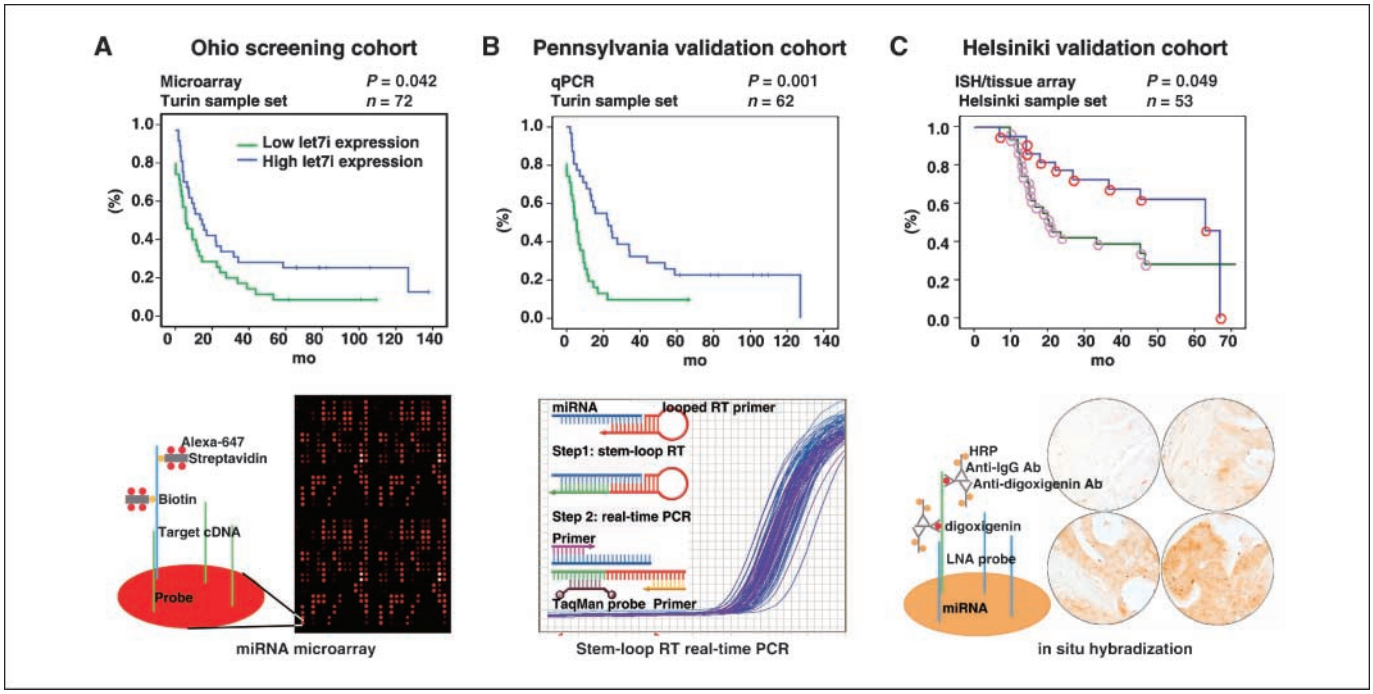


Figure 4. Low *let-7i* expression is significantly associated with shorter survival of patients with EOC. Correlation between *let-7i* expression and survival of EOC patients analyzed by microarray (*A,* progression-free survival), real-time reverse transcription-PCR (*B,* progression-free survival), and tissue array (*C,* disease-free survival).

cycle–associated genes, e.g., CDC25A (22), CDK6 (22), CDK4 (39), and cyclin A (39), cyclin D1 (39), cyclin D2 (22), and cyclin D3 (39). Finally, let-7 regulates a panel of oncofetal genes, e.g., IMP-1/CRD-BP (40), and Toll-like receptors, e.g., TLR4 (38). Consistent with other investigators' reports (17, 23–25, 27, 28), we also found that overexpression of *let-7i* was able to remarkably down-regulate oncogenic proteins such as H-RAS and HMGA2, in EOC cell lines (data not shown). Up to one-third of human mRNAs seem to be miRNA targets (41). Each miRNA can target hundreds of transcripts directly or indirectly (42, 43), and more than one miRNA can converge on a single transcript target (44). Thus, the potential regulatory circuitry afforded by the *let-7* family is enormous. We would not expect that one or even a few target proteins play a key role in *let-7i*'s function of chemotherapy sensitivity. Instead, we believe that there is a complex molecular network involved in this function (Fig. 5*A*), which indicates that restoring *let-7i* might be a more efficient strategy compared with only targeting one protein-coding gene to modulate chemotherapy because multiple pathways will be affected by *let-7i*–based therapy.

Recently, an interesting role of the *let-7* family in self-renewing progenitor cells has been reported (27, 45). Ibarra and colleagues found that *let-7* is depleted in the mouse mammary epithelial cell line, comma-Dβ, which contains a population of self-renewing progenitor cells that can reconstitute the mammary gland, suggesting its role in the regulation of progenitor maintenance (45). Self-renewing tumor-initiating cells (T-IC) or cancer stem cells have been identified and implicated to give rise to cancer. By comparing miRNA expression in self-renewing and differentiated cells from breast cancer lines and in breast T-IC (BT-IC) and non–BT-IC from first-degree breast cancers, Yu and colleagues found that the *let-7* family was markedly reduced in BT-IC and increased with differentiation (27). They also showed that the *let-7* family regulates multiple BT-IC stem cell–like properties and tumorigenicity of breast cancer cells by silencing more than one target including H-RAS and HMGA2 (27). Those findings are particularly important to our understanding of the role of *let-7* family in cancer, and especially in patient response to chemotherapy, because chemotherapy selectively enhances the proportionate survival of BT-IC (27). In addition, those results strongly suggest that *let-7*–based targeted therapy might more efficiently differentiate the chemotherapy-resistant cancer stem cell population (Fig. 5*B*).

Although the *let-7* family has been generally shown as a tumor suppressor gene, there have been contradictory reports that certain members of the *let-7* family could also serve an oncogenic function. For example, *let-7a-3* has been reported to be located in a CpG locus by two groups (21, 30), and we also reported that the chromosome region harboring this miRNA is highly deleted in EOC (7). However, Lu and colleagues reported that hypermethylation of *let-7a-3* in EOC is associated with a favorable prognosis (21), whereas Brueckner and colleagues made the opposite discovery that its hypomethylation results in enhanced tumor phenotype in cultured HCT 116 colon cancer cells (30). Those results indicated that the highly homologous *let-7* family members may play contradicting functions in different cancer types or cellular context. Most recently, Vasudevan and colleagues documented that *let-7* induces translation up-regulation of target mRNAs on cell cycle arrest, yet it represses translation in proliferating cells (46). Therefore, the function of *let-7* family in cancer might be more complex than we previously expected before. Further studies are needed on the function of individual *let-7* family members in human cancer.

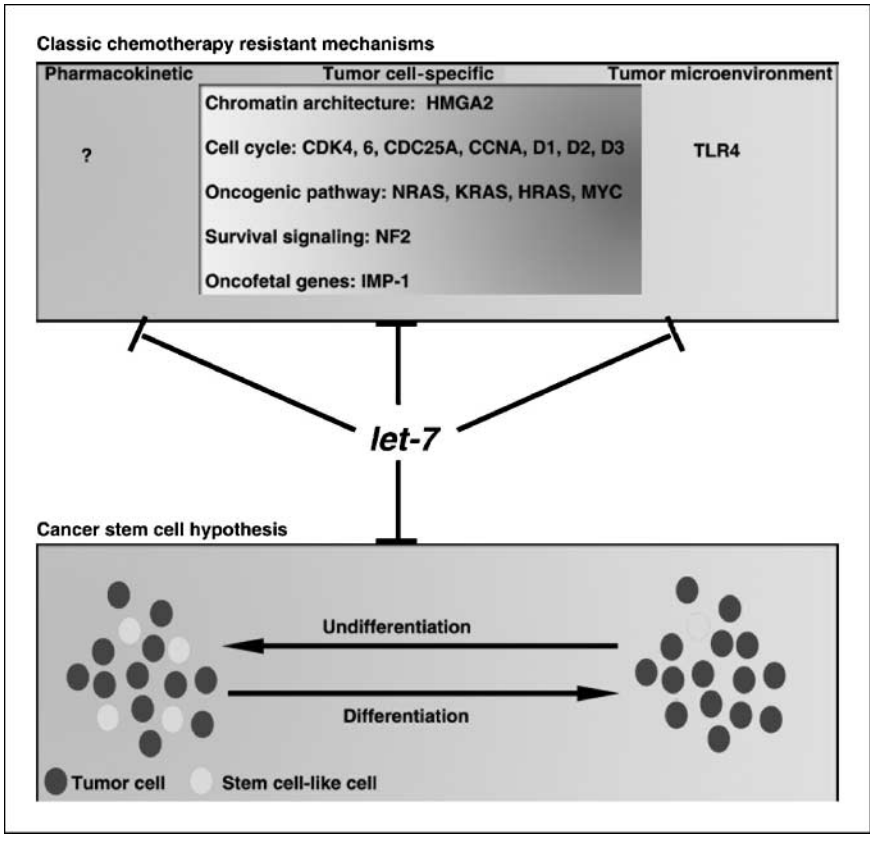


Figure 5. Illustration of the potential mechanism of *let-7i* regulating chemotherapy sensitivity in human cancer.

miRNAs provide a therapeutic target for cancer treatment (5). Modified antisense oligonucleotides complementary to miRNAs are used by many groups to inhibit miRNAs with oncogenic properties. To supplement and/or enhance the function of tumor suppressor miRNAs, enforced expression of a short hairpin RNA from a polymerase II or III promoter in a nonviral or viral vector, which can be further processed into mature miRNAs, has been tested (47–50). In addition, *in vivo* delivery of double-stranded miRNA mimics has been reported (47–50). The current rapid advances in oligonucleotide/nanoparticle therapy create realistic optimism for the establishment of the *let-7* family as a new and potent therapeutic target and/or chemoresistant modulator in cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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