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Clin Cancer Res 2008;14:2690-2695.

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Abstract

Purpose: Although microRNAs have recently been recognized as riboregulators of gene expression, little is known about microRNA expression profiles in serous ovarian carcinoma. We assessed the expression of microRNA and the association between microRNA expression and the prognosis of serous ovarian carcinoma.

Experimental Design: Twenty patients diagnosed with serous ovarian carcinoma and eight patients treated for benign uterine disease between December 2000 and September 2003 were enrolled in this study. The microRNA expression profiles were examined using DNA microarray and Northern blot analyses.

Results: Several microRNAs were differentially expressed in serous ovarian carcinoma compared with normal ovarian tissues, including *miR-21, miR-125a, miR-125b, miR-100, miR-145, miR-16*, and *miR-99a*, which were each differentially expressed in >16 patients. In addition, the expression levels of some microRNAs were correlated with the survival in patients with serous ovarian carcinoma. Higher expression of *miR-200, miR-141, miR-18a, miR-93*, and *miR-429*, and lower expression of *let-7b*, and *miR-199a* were significantly correlated with a poor prognosis ($P < 0.05$).

Conclusion: Our results indicate that dysregulation of microRNAs is involved in ovarian carcinogenesis and associated with the prognosis of serous ovarian carcinoma.

Ovarian cancer is the most lethal of the gynecologic malignancies, but relatively little is known about the molecular genetics of its initiation and progression. Epithelial ovarian cancer, which accounts for 90% of ovarian cancer, is a heterogeneous group of neoplasms and is divided into histologic subgroups, each with their own underlying molecular genetic events: serous, mucinous, endometrioid, clear cell, Brenner, and undifferentiated carcinomas (1, 2). Among them, the serous type accounts for 75% to 80% of epithelial ovarian carcinomas. High grade serous ovarian carcinoma has a high incidence of *TP53* (3), *HER-2/ERBB2* (4), and *AKT2* gene mutations (5) but a low incidence of *KRAS* and *BRAF* gene mutations (6). Nevertheless, the primary genetic alterations associated with serous ovarian carcinoma remain to be identified.

MicroRNAs (miRNA) are noncoding, single-stranded RNAs of ~22 nucleotides in length that constitute a novel class of gene regulators. miRNAs function as guide molecules by base paring with the mRNAs partially complementary to the miRNAs in miRNA-associated effector complexes (7). The binding of miRNAs to their target mRNAs leads to translational repression or decreases the stability of the mRNA (7, 8). miRNAs control various biological processes, including cell differentiation, cell proliferation, apoptosis, stress resistance, and fat metabolism (9).

Some miRNAs possess oncogenic or tumor suppressor activity. The first study documenting abnormalities in miRNA expression in tumors identified *miR-15a* and *miR-16-1*, which are located in a frequently deleted region in B-cell chronic lymphocytic leukemia (10). In a subsequent study, the miRNAs were found to suppress *BCL2*, an antiapoptic gene (11). The *let-7* family, which is down-regulated in lung cancer when *RAS* is frequently mutated (12), negatively regulates *RAS* (13). *miR-155* is overexpressed in Burkitt's lymphoma (14), breast cancer (15), and lung cancer (16). Its overexpression in transgenic mice leads to preleukemic pre–B-cell proliferation (17). *miR-372* and *miR-373* cooperate with oncogenic *RAS* in the cellular transformation of testicular germ cell tumors (18). All these findings suggest that altered miRNA expression contributes to tumorigenesis.

miRNA expression profiles show unique expression patterns according to the clinical features in several cancers, including chronic lymphocytic leukemia (19), breast cancer (15), pancreatic cancer (20), and lung cancer (12, 16), suggesting that some miRNAs could be used as diagnostic and prognostic markers. Of note, Lu et al. (21) found that miRNA expression profiling is much better for classifying poorly differentiated samples than mRNA expression profiling. Therefore, miRNA profiling may have a crucial clinical application.

In this study, we investigated the miRNA expression profiles in serous ovarian carcinoma and attempted to identify miRNAs capable of predicting the clinical prognosis.

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Received 7/13/07; revised 11/5/07; accepted 12/20/07.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-07-1731

Materials and Methods

Patients and tumor tissues. After obtaining informed consent and performing surgery at the Yonsei University College of Medicine (Seoul, Korea), samples of primary epithelial ovarian cancer were snap frozen in liquid nitrogen and stored at -80°C. The histopathologic diagnoses were determined using the WHO criteria, and the tumor histotype was serous cystadenocarcinoma in all patients. Twenty-eight samples were used for this study, including 20 serous ovarian carcinomas and 8 normal ovarian tissues. The clinical data and patient information are shown in Supplementary Table S1. Nine patients whose cancer progressed within 12 mo from the initiation of platinum-based combination chemotherapy were defined as the chemoresistant-disease group, whereas 11 patients whose cancer did not recur for >24 mo were defined as the chemosensitive-disease group. Progression was defined as the appearance of a new metastatic site that was not present at the initiation of primary chemotherapy or an abnormal CA-125 value with an increase of >25% over the previous level. Progression-free survival was defined as the interval from the initiation date of primary chemotherapy to the date of progression. Overall survival was calculated from the initiation date of primary chemotherapy until the date of death or the last follow-up visit.

miRNA microarrays. The frozen samples were homogenized in TRIzol reagent (Invitrogen) using an Omni-Mixer Homogenizer (Omni International). Total RNA was then isolated according to the manufacturer's instructions. Total RNA from eight normal ovarian tissues was pooled and used as a common reference for miRNA expression. For the miRNA microarray study, 50 μg of total RNA were further processed to enrich the miRNA using a Purelink miRNA Isolation kit (Invitrogen). DNA oligonucleotide probes from the mirVana miRNA Probe Set (Ambion), which contains 314 human, 49 mouse, and 14 rat miRNA genes, were printed on coated glass slides in duplicate (Digital Genomics); 50 μmol/L probes were resuspended with 3× SSC and spotted on AttayIt SuperEpoxy2 (TeleChem) under 55% humidity using the ArrayIt SpotBot (TeleChem). The spot diameter was 100 μm. The slides were rehydrated and blocked in a solution containing 100 mmol/L ethanolamine, 1 mol/L Tris (pH 9.0), and 0.1% SDS for 20 min at 50°C and then rinsed thoroughly with water and spun dry. Purified miRNAs were labeled using a mirVana miRNA Labeling kit (Ambion) and amine-reactive Cy5 or Cy3 dyes, as recommended by the manufacturer. Poly(A) polymerase and a mixture of unmodified and amine-modified nucleotides were first used to append a polynucleotide tail to the 3′ end of each miRNA. The amine-modified miRNAs were then cleaned and coupled to NHS-ester–modified Cy5 or Cy3 dye (Amersham Biosciences). The RNA from normal ovarian tissues and cancer tissues was labeled with Cy3 and Cy5 dye, respectively. Slides were hybridized for 12 to 16 h at 42°C in sealed cassettes under controlled humidity.

Data analysis. After hybridization, the slides were washed and dried before performing a high-resolution scan on a GenePix 4000B Array Scanner (Axon Instruments). The scanned images were analyzed with GenePix software version 3.0 (Axon Instruments) to obtain gene expression ratios. Transformed data were normalized using the Lowess procedure (Supplementary Fig. S1; ref. 22) and subjected to analysis using the Significance Analysis of Microarrays software program (SAM, Version 1.10) and clustering analysis (23). The clustering analysis was done using the Cluster and TreeView programs available online.[4] Abnormal data were flagged during the GenePix software analysis and these numerical data were excluded from the SAM analysis. Missing data were input using the average of 10 nearest neighbors. For the clinical data analysis, only the miRNAs differentially expressed in more than three patients were selected to decrease possible technical errors. Fisher's exact test and the Mann-Whitney *U* test were used to evaluate which miRNAs could discriminate the chemosensitive and chemoresistant groups (ver. 12.0; SPSS, Inc.). For the selected miRNAs, receiver operating characteristics curve analysis was used to assess the diagnostic accuracy of each miRNA. Survival analysis was used to compare patients with or without specific miRNAs in terms of progression-free and overall survival. The Kaplan-Meier method was used to estimate survival curves, and the log-rank statistic was used to test the equality of the survival functions between the patients with or without specific miRNA.[4]

Northern blot analysis. Twenty-microgram RNA samples were separated on 15% Tris-borate EDTA urea acrylamide gels (Bio-Rad), and then transferred onto Hybond-N+ membranes (Amersham Biosciences) and subjected to UV cross-linking. The oligonucleotides with a sequence complementing the mature miRNAs were labeled using polynucleotide kinase and [γ-^{32}P]ATP. The miRNAs were hybridized in ULTRAhyb-oligo (Ambion) at 37°C overnight. The membranes were washed thrice with 2× SSC + 0.1% SDS for 5 min at room temperature then exposed to a phosphor screen for 24 to 72 h and imaged using a BAS-2500 (Fujifilm). The signal intensities were measured using the program TINA2.0 (TINA). The blots were stripped with 0.1% SSC + 0.1% SDS at 80°C for 30 min and were reprobed. 5S RNA was used as a loading control.

Table 1. Differentially expressed miRNAs with >2-fold change in tumor versus normal ovarian tissues in at least 12 of 20 ovarian cancer patients

miRNA	Up*	(%)	Down*	(%)	Mean† M ± SD (median)
miR-145	0/20	0	17/20	85	-3.20 ± 1.13 (-3.38)
miR-125b	0/20	0	19/20	95	-2.74 ± 0.80 (-2.59)
miR-100	0/20	0	18/20	90	-2.57 ± 1.08 (-2.47)
miR-99a	0/20	0	18/20	90	-2.48 ± 0.93 (-2.37)
miR-26a	0/20	0	15/20	75	-2.17 ± 0.78 (-1.92)
miR-10b	1/20	5	12/20	60	-1.99 ± 1.34 (-1.87)
miR-143	1/20	5	14/20	70	-1.70 ± 1.06 (-1.85)
miR-214	0/20	0	12/20	60	-2.08 ± 0.93 (-1.81)
let-7b	1/20	5	13/20	65	-1.57 ± 1.06 (-1.63)
miR-199a-AS	2/20	10	11/20	55	-1.36 ± 1.34 (-1.48)
miR-29a	1/20	5	12/20	60	-1.50 ± 1.15 (-1.46)
miR-125a	2/20	10	16/20	80	-1.18 ± 0.97 (-1.26)
miR-93	14/20	70	0/20	0	1.68 ± 0.53 (1.56)
miR-23b	11/20	55	1/20	5	1.63 ± 1.63 (2.02)
miR-20a	12/20	60	0/20	0	2.12 ± 0.65 (2.10)
miR-27a	12/20	60	1/20	5	1.85 ± 1.16 (2.22)
miR-16	16/20	80	1/20	5	2.49 ± 1.65 (2.29)
miR-23a	11/20	55	1/20	5	1.77 ± 1.52 (2.36)
miR-200a	12/20	60	0/20	0	2.58 ± 0.79 (2.71)
miR-200b	14/20	70	0/20	0	2.84 ± 0.92 (2.82)
miR-21	17/20	85	0/20	0	2.89 ± 1.20 (2.88)
miR-200c	14/20	70	0/20	0	3.19 ± 0.95 (3.00)
miR-141	14/20	70	0/20	0	2.91 ± 0.87 (3.02)

*Frequency of up or down-regulated miRNAs, which were considered differentially expressed if their M values are >1.0 or lower than (-)1.0.
† M, value of $\log_2$ (Cy5/Cy3).

Results

Distinct miRNA signatures in serous ovarian cancer, compared with normal ovarian tissues. To identify miRNAs differentially expressed in serous ovarian cancer compared with the corresponding normal tissues, we used a customized miRNA microarray that contained 314 human miRNAs from the miRNA Registry (24). We analyzed the miRNA expression

[4] http://rana.lbl.gov

profiles of 20 serous ovarian cancer tissues using a two-color system. We used pooled miRNAs from normal tissues as a common reference for each cancer case. After normalization, an "MA plot" was used to represent the data, where M = $\log_2$ (Cy5/Cy3). The confidence of the signal was confirmed using SAM analysis (23). miRNAs were considered differentially expressed if their M values were >1.0 or <-1.0. Twenty-three miRNAs were differentially expressed in at least 12 of the 20 serous ovarian cancers (Table 1). The up-regulated miRNAs (n = 11) were *miR-200a*, *miR-200b*, *miR-200c*, *miR-20a*, *miR-21*, *miR-23a*, *miR-23b*, *miR-27a*, *miR-141*, *miR-16*, and *miR-93*. The down-regulated miRNAs (n = 12) were *miR-214*, *miR-26a*, *miR-29a*, *let-7b*, *miR-100*, *miR-10b*, *miR-125a*, *miR-125b*, *miR-143*, *miR-145*, *miR-199a-AS*, and *miR-99a*. Of these miRNAs, the most frequently up-regulated miRNA was *miR-21* (17 of 20 cases), and the most frequently down-regulated miRNA was *miR-125b* (19 of 20 cases). Although the cancer specimens were obtained from different patients, the expression trends of the miRNAs were homogenous across almost all cases. miRNA families displaying one or two base differences, such as *miR-200a*, *miR-200b*, and *miR-200c*, showed similar expression profiles in the same ovarian cancer patients (Fig. 1).

Validation of the microarray results. To validate the microarray results, Northern blots of the differentially expressed miRNAs were done independently. We chose *miR-200c*, *miR-93*, *miR-141*, *let-7b*, *miR-99a*, and *miR-125b*, which were differentially expressed between the normal ovarian tissues and serous ovarian cancer tissues (Fig. 2). In agreement with the microarray results, *miR-200c*, *miR-93*, and *miR-141* were up-regulated, whereas *let-7b*, *miR-99a*, and *miR-125b* were down-regulated in ovarian carcinoma. We quantified the band intensity of each miRNA and calculated the relative expression ratios after normalizing each band intensity to 5S RNA. The average correlation between the Northern blot results and microarray data were 0.791 (P < 0.001; Supplementary Fig. S2). We found that *let-7b* had a lower correlation between the two analyses compared with the other miRNAs tested, which might have resulted from the high ability of the *let-7* family to cross-hybridize, as reported previously (21). Overall, however, the microarray data were good enough to warrant further analyses in a clinical setting.

miRNA signatures are associated with the prognosis of serous ovarian carcinoma. Next, we wanted to determine whether the miRNA expression profiles could distinguish chemosensitive cases from chemoresistant cases to assess the effect of miRNA expression on prognosis. The majority of the cases were separated into the two groups using an unsupervised hierarchical cluster analysis (Fig. 1). However, seven cases did not separate well based on the gene expression profiles of the selected miRNAs. Therefore, Fisher's exact test was done using the binomial variable whether the miRNA was differentially expressed or not. *miR-199a* and *ambi-miR-7039* were significantly down-regulated in the chemoresistant group (P < 0.05; Supplementary Table S2). *Ambi-miR-7039* are newly identified human miRNAs called Ambi-miR miRNAs. Based on the sequences provided by the company, *ambi-miR-7039* is a small nucleolar RNA. Therefore, we excluded *ambi-miR-7039* from

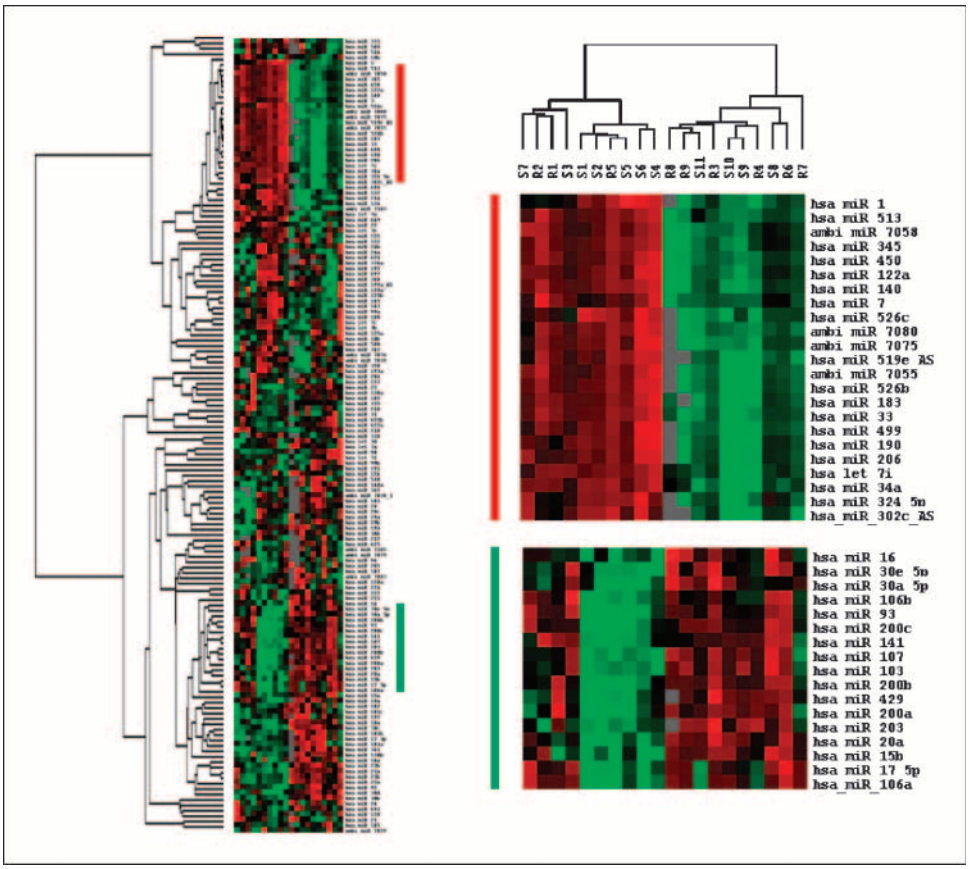

Fig. 1. Unsupervised hierarchical clustering analysis of miRNAs that exhibited a >2-fold increase or decrease in at least three cases of serous ovarian carcinoma. The level of miRNA expression is color-coded. *Red,* higher miRNA expression; *green,* lower miRNA expression; *black,* no difference. S, chemosensitive disease; R, chemoresistant disease.

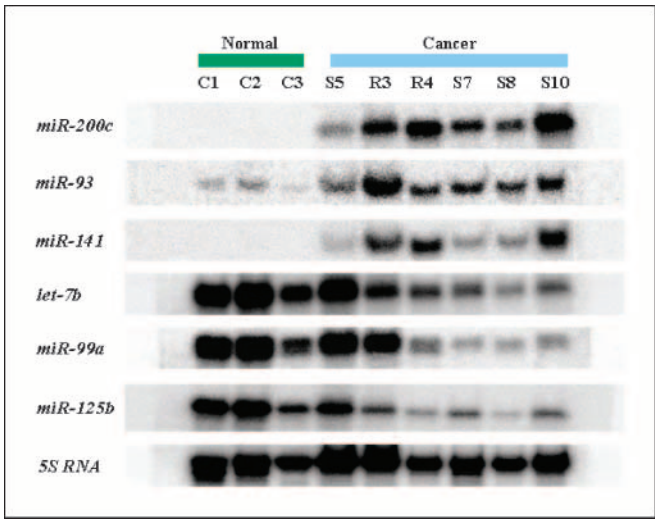

Fig. 2. Northern blot of selected miRNAs. *miR-200c, miR-93*, and *miR-141* were up-regulated, whereas *let-7b, miR-99a*, and *miR-125b* were down-regulated in serous ovarian carcinomas, in agreement with the microarray results. C1-C3, three different normal ovarian tissues; the same samples were pooled as a common reference in the microarray experiment. S, chemosensitive disease; R, chemoresistant disease.

further analysis. To evaluate the usefulness of miRNAs for detecting chemoresistant disease, we did a receiver operating characteristics analysis on the selected miRNAs to detect the recurrence of persistent ovarian cancer. Down-regulated *miR-199a* had the greatest area under the curve (0.763; Supplementary Table S2). As a result, the down-regulation of *miR-199a* could be a reliable marker for predicting chemoresistant disease.

Second, we investigated the correlation between the miRNA expression profile and survival. Kaplan-Meier analysis was done, and the median survival based on the expression of miRNA is shown in Table 2. Higher expression of *miR-200a, miR-200b, miR-200c, miR-141, miR-18a, miR-93*, and *miR-429*, and lower expression of *ambi-miR-7039, let-7b*, and *miR-199a* were significantly correlated with decreased progression-free survival and overall survival (log-rank test, $P < 0.05$; Table 2). As shown in Fig. 3, tumors with high expression of *miR-200a* had a median overall survival of 27.5 months (95% confidence interval, 22.8-46.0) compared with 61.0 months (95% confidence interval, 43.5-67.8) for those with no significant expression ($P = 0.0054$).

Discussion

We identified 23 miRNAs whose expression is significantly deregulated in serous ovarian carcinoma compared with normal ovarian tissues. These miRNAs may be used to distinguish serous ovarian cancer from normal ovarian tissue. While our article was being reviewed, a study of miRNA expression profiling in ovarian cancer was published by Iorio and colleagues (25). Half of the miRNAs that we identified (Table 1) are seen in their list of miRNAs aberrantly expressed. They include the most significantly altered miRNAs in both studies: *miR-200a/b/c, miR-141, miR-21, miR-145, miR-99a, let-7*, and *miR-125b*. This concordance further supports our findings and relevance of these miRNAs to ovarian cancer.

The most consistently up- and down-regulated miRNAs were *miR-21* and *miR-125b*, respectively. *miR-21* is also up-regulated in other cancers such as glioblastoma (26), breast cancer (15), and cholangiocarcinoma cell lines (27). In recent reports, the up-regulation of *miR-21* was associated with an antiapoptotic effect on tumor growth (27). The inhibition of *miR-21* using antisense oligonucleotides increased the expression level of PTEN (27), a negative regulator of the phosphatidylinositol-3-OH kinase–AKT signaling pathway, and decreased *Bcl-2* (28), an antiapoptotic gene, resulting in inducing apoptosis. A loss of heterozygosity of *PTEN* was seen in ~30% of serous ovarian cancer without additional somatic mutations (29). Serous ovarian cancer may acquire an antiapoptotic phenotype by inactivating *PTEN* genetically and up-regulation of *miR-21*.

miR-125b was implicated in differentiation (30), cell proliferation, and mobility (31). Receptor tyrosine kinases *ERBB2* and *ERBB3* were shown to be targets of *miR-125*a/b (31). Overexpression of *miR-125*a/b in an ERBB2-positive breast cancer cell line impaired cell growth and mobility

Table 2. Median survival of serous ovarian carcinoma patients according to the miRNA expression profiling

	Median progression-free survival ± SD (mo)			Median overall survival ± SD (mo)		
	Significant expression*	No significant expression	*P* †	Significant expression*	No significant expression	*P* †
miR-200a	11.0 ± 20.7	54.5 ± 24.8	0.0302	27.5 ± 18.3	61.0 ± 14.5	0.0054
miR-200b	11.0 ± 22.9	54.5 ± 20.5	0.0183	29.5 ± 18.8	61.0 ± 11.2	0.0334
miR-200c	11.0 ± 22.9	54.5 ± 20.5	0.0163	29.5 ± 18.8	61.0 ± 11.2	0.0183
miR-27a	12.0 ± 22.0	55.0 ± 25.2	0.0677	30.0 ± 18.2	57.0 ± 17.2	0.0669
miR-31	9.5 ± 11.9	45.0 ± 25.2	0.1118	26.5 ± 18.7	53.5 ± 18.7	0.1104
ambi-miR-7039	7.5 ± 15.6	53.5 ± 21.7	0.0059	27.0 ± 19.5	55.5 ± 15.5	0.0136
let-7b	11.0 ± 24.4	54.0 ± 21.9	0.0194	29.5 ± 18.8	57.0 ± 7.0	0.0149
miR-141	11.0 ± 23.0	54.5 ± 20.5	0.0163	29.5 ± 18.8	61.0 ± 11.2	0.0183
miR-18a	10.5 ± 18.1	46.0 ± 25.7	0.0472	27.5 ± 17.2	55.5 ± 19.1	0.0345
miR-182	10.0 ± 21.8	40.0 ± 25.2	0.1104	25.0 ± 16.7	56.0 ± 18.1	0.0575
miR-199a	9.5 ± 21.8	54.0 ± 20.1	0.0164	27.0 ± 20.4	56.5 ± 11.7	0.0181
miR-429	9.0 ± 17.3	52.0 ± 23.9	0.0083	29.0 ± 17.7	55.0 ± 18.2	0.0186
miR-93	11.0 ± 23.0	54.5 ± 20.5	0.0163	29.5 ± 18.8	61.0 ± 11.2	0.0183

*Significant expression of miRNA was defined if M value >1 or <-1.
† Log-rank test gave significant *P* values ($P < 0.05$).

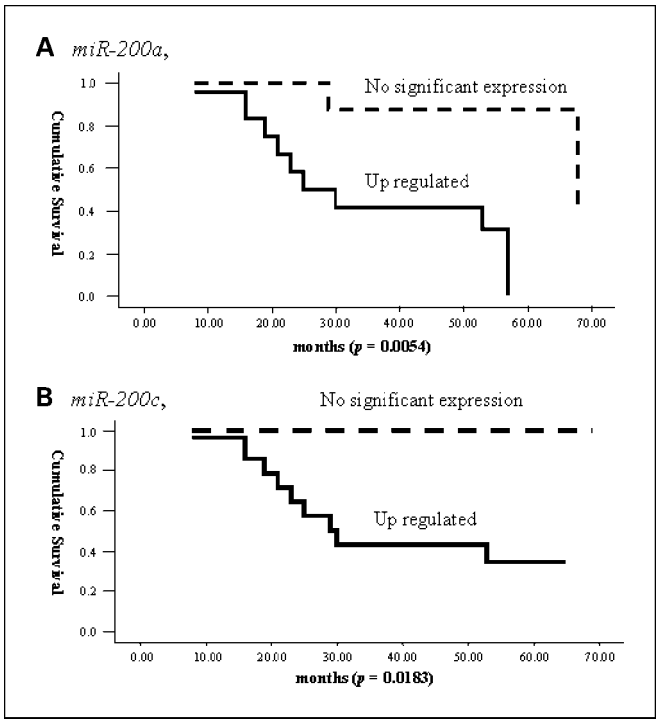

Fig. 3. Kaplan-Meier overall survival curve for patients with serous ovarian carcinoma based on the expression of miRNAs. The higher expression of *miR-200a* (*A*) and *miR-200c* (*B*) was significantly correlated with a shorter overall survival. The log-rank test gave significant *P* values ($P < 0.05$).

capability (31). Frequent overexpression or amplification of *ERBB2* was reported in ~20% of serous ovarian cancers (2, 32). Because the chromosome 11q23-24 for *miR-125b-1* is one of the regions most frequently deleted in breast, ovarian, and lung cancers (33), correlation between the overexpression of *ERBB2* or *ERBB3* and loss of *miR-125a/b* loci might exist. In addition to *miR-125b*, low levels of *let-7* were seen frequently in our cases. Some of them might be related to oncogenic mutations in *KRAS*, accounting for 35% of low-grade serous ovarian carcinoma (2), based on reciprocal relationship between *let-7* and its target gene *RAS* (13). In addition to oncogenes *ERBB2*, *BRBB3*, and *RAS*, additional target genes of *miR-125b* and *let-7* might be responsible for the high frequency of their down-regulation in serous ovarian cancer.

Contrary to a previous report (11), we observed increased levels of *miR-15* and *miR-16*, which are miRNAs with tumor-suppressing properties. Their lower levels were associated with high antiapoptotic BCL2 protein in chronic lymphocytic leukemia (11). This discrepancy could have resulted from the activity of specific miRNAs in different tissues or cellular contents (34). For example, the *miR-17* cluster was amplified in human non–small cell lung cancer (35), whereas the cluster was frequently deleted in hepatocellular carcinoma (36).

Our findings suggest that the most reliable predictive marker for chemoresistant disease was the down-regulation of *miR-199a* (area under the curve, 0.763), which is located on chromosome 19p13.2. Web-based computational approaches (TargetScan, PicTar-Vert, and MiRanda) predict hundreds of potential targets of *miR-199a*, including discoidin domain receptor family member 1 (*DDR1*), a gene predicted by at least two of these methods that contains more than three conserved sites for the miRNA. DDR1 is a receptor tyrosine kinase activated by various collagens, and it plays a role in cell attachment, migration, survival, and proliferation. DDR1 was reported to induce cyclooxygenase-2 expression resulting in enhanced chemoresistance via the nuclear factor-κB pathway (37). However, further studies are needed to validate the function of this miRNA.

Ten miRNAs were significantly correlated with patient survival. *miR-199a* was also related to patient survival because clinically, survival might be affected markedly by chemoresistance. Notably, of the identified miRNAs (Table 2), five miRNAs originated from two clustered chromosome regions: *miR-200a*, *miR-200b*, and *miR-429* are from chromosome 1p36.33, and *miR-200c* and *miR-141* are from chromosome 12p13.31. The increased expression of *miR-200a* might underlie genomic amplification. Frequent chromosomal gains in that region have been reported in serous ovarian carcinoma (38, 39). The inhibition of *miR-200b* increased the sensitivity to gemcitabine in cholangiocarcinoma cell lines (27), and thereby, increased expression of *miR-200b* may result in poor response to antineoplastic drugs. Moreover, inhibition of *miR-141* using anti–miR-141 decreased cell growth in cholangiocarcinoma cell lines (27). Accordingly, these miRNAs could be a novel target for enhancing chemosensitivity or inducing cell death in serous ovarian cancer. As a result, we can predict the prognosis of patients with serous ovarian carcinoma using the deregulated miRNAs. Although other studies have examined miRNAs in ovarian cancer (21, 25, 39), to our knowledge, ours is the first to ascribe a prognostic value to miRNAs expression profiles in serous ovarian carcinoma.

Overall, our report contributes to the understanding of miRNA expression profiling and its relationship to tumorigenesis in serous ovarian carcinoma. Moreover, some miRNAs are significantly correlated with the response to chemotherapy and survival in serous ovarian carcinoma, and could be used as prognostic markers.

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