MicroRNA Signatures in Human Ovarian Cancer

Marilena V. Iorio,[1] Rosa Visone,[2] Gianpiero Di Leva,[2] Valentina Donati,[2] Fabio Petrocca,[2] Patrizia Casalini,[1] Cristian Taccioli,[2] Stefano Volinia,[2] Chang-Gong Liu,[2] Hansjuerg Alder,[2] George A. Calin,[2] Sylvie Ménard,[1] and Carlo M. Croce[2]

[1]Molecular Biology Unit, Department of Experimental Oncology, Fondazione Istituti di Ricovero e Cura a Carattere Scientifico, Istituto Nazionale Tumori, Milano, Italy and [2]Department of Molecular Virology, Immunology and Medical Genetics and Comprehensive Cancer Center, Ohio State University, Columbus, Ohio

Abstract

Epithelial ovarian cancer (EOC) is the sixth most common cancer in women worldwide and, despite advances in detection and therapies, it still represents the most lethal gynecologic malignancy in the industrialized countries. Unfortunately, still relatively little is known about the molecular events that lead to the development of this highly aggressive disease. The relatively recent discovery of microRNAs (miRNA), a class of small noncoding RNAs targeting multiple mRNAs and triggering translation repression and/or RNA degradation, has revealed the existence of a new level of gene expression regulation. Multiple studies involving various types of human cancers proved that miRNAs have a causal role in tumorigenesis. Here we show that, in comparison to normal ovary, miRNAs are aberrantly expressed in human ovarian cancer. The overall miRNA expression could clearly separate normal versus cancer tissues. The most significantly overexpressed miRNAs were *miR-200a, miR-141, miR-200c*, and *miR-200b*, whereas *miR-199a, miR-140, miR-145*, and *miR-125b1* were among the most down-modulated miRNAs. We could also identify miRNAs whose expression was correlated with specific ovarian cancer biopathologic features, such as histotype, lymphovascular and organ invasion, and involvement of ovarian surface. Moreover, the levels of *miR-21, miR-203*, and *miR-205*, up-modulated in ovarian carcinomas compared with normal tissues, were significantly increased after 5-aza-2′-deoxycytidine demethylating treatment of OVCAR3 cells, suggesting that the DNA hypomethylation could be the mechanism responsible for their overexpression. Our results indicate that miRNAs might play a role in the pathogenesis of human EOC and identify altered miRNA gene methylation as a possible epigenetic mechanism involved in their aberrant expression. [Cancer Res 2007;67(18):8699–707]

Introduction

Epithelial ovarian cancer (EOC) is the most common gynecologic malignancy and the sixth most common cancer in women worldwide, with highly aggressive natural history causing almost 125,000 deaths yearly (1). Despite advances in detection and cytotoxic therapies, only 30% of patients with advanced-stage ovarian cancer survive 5 years after initial diagnosis (2). The high mortality of this disease is mainly due to late-stage diagnosis for >70% of ovarian cancers. In fact, when ovarian cancer is diagnosed in its early stage, that is still organ confined, the 5-year survival rate exceeds 90%. Unfortunately, only 19% of all ovarian cancers are diagnosed at this early stage. Indeed, this rather poor prognosis is due to (*a*) the insidious asymptomatic nature of this disease in its early onset, (*b*) the lack of robust and minimally invasive methods for early detection, and (*c*) tumor resistance to chemotherapy. The vast majority of human ovarian carcinomas are represented by ovarian epithelial cancers (OEC), deriving from the ovarian surface epithelium (3).

Ovarian adenocarcinomas occur as four major histologic subtypes, serous, mucinous, endometrioid, and clear cell, with serous being the most common. Current data indicate that each of these histologic types is associated with distinct morphologic and molecular genetic alterations (4), but further investigations of the molecular mechanisms promoting ovarian cancer are necessary to determine how each of the subtypes emerges.

Over the last 5 years, expression profiling technologies greatly improved, thus expanding the knowledge on cancer etiology and biomarkers with clinical applications (5, 6). However, although these technologies have provided most of the new biomarkers with potential use for diagnosis, drug development, and tailored therapy, they have thus far shed little insight into the detailed mechanisms at the origin of this neoplasia, thus suggesting that ovarian tumorigenesis may occur through novel or poorly characterized pathways.

A new class of small noncoding RNAs, named microRNAs (miRNA), was discovered recently and shown to regulate gene expression at post-transcriptional level, for the most part by binding through partial sequence homology to the 3′ untranslated region of target mRNAs and causing block of translation and/or mRNA degradation (7). miRNAs are 19 to 25 nt long molecules cleaved from 70 to 100 nt hairpin pre-miRNA precursors. The precursor is cleaved by cytoplasmic RNase III Dicer into ~22 nt miRNA duplex: one strand (miRNA*) of the short-lived duplex is degraded, whereas the other strand, which serves as mature miRNA, is incorporated into the RNA-induced silencing complex and drives the selection of target mRNAs containing antisense sequences.

Several studies have shown that miRNAs play important roles in essential processes, such as differentiation, cell growth, and cell death (8, 9).

Moreover, it has been shown that miRNAs are aberrantly expressed or mutated in cancers, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate: *let-7*, down-regulated in lung cancer, suppresses RAS (10) and HMGA2 (11, 12); *mir-15* and

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

Requests for reprints: Carlo M. Croce, Department of Molecular Virology, Immunology and Medical Genetics and Comprehensive Cancer Center, Ohio State University, Room 445C, Wiseman Hall, 400 12th Avenue, Columbus, OH 43210. Phone: 614-292-3063; Fax: 614-292-3312; E-mail: Carlo.Croce@osumc.edu.

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mir-16, deleted or down-regulated in leukemia, suppress BCL2 (13); and *mir-17-5p* and *mir-20a*, control the balance of cell death and proliferation driven by the proto-oncogene c-Myc (14). Clear evidences indicate that miRNA polycistron *mir-17-92* acts as an oncogene in lymphoma and lung cancer (15); *mir-372* and *mir-373* are novel oncogenes in testicular germ cell tumors by numbing p53 pathway (16); *miR-155*, overexpressed in B-cell lymphomas and solid tumors, leads to the development of B-cell malignancies in an *in vivo* model of transgenic mice (17).

The use of miRNA microarray technologies has been used as a powerful tool to recognize miRNAs differentially expressed between normal and tumor samples (18–20) and also to identify miRNA expression signatures associated with well-defined clinico-pathologic features and disease outcome (21, 22). Several studies have also investigated the molecular mechanisms leading to an aberrant miRNA expression, identifying the presence of genomic abnormalities in miRNA genes (21, 23, 24). More recently, few evidences have shown that miRNA genes may be regulated also by epigenetic mechanisms, as changes in genomic DNA methylation pattern: *miR-127* (25) and *miR-124a* (26) are transcriptionally inactivated by CpG island hypermethylation, whereas in lung cancer, the overexpression of *let-7a-3* seems to be due to DNA hypomethylation (27).

Here, we present the results of a genome-wide miRNA expression profiling in a large set of normal and tumor ovarian tissues, showing the existence of an ovarian cancer-specific miRNA signature and identifying the altered methylation of miRNA genes as a possible epigenetic mechanism responsible for their aberrant expression.

Materials and Methods

Ovarian cancer samples and cell lines. A total of 84 snap-frozen normal and malignant ovarian tissues were collected at the GOG Tissues Bank, Columbus Children's Hospital (Columbus, OH). The tissue collection used for microarray analysis included 15 normal ovarian tissue sections and 69 malignant tissues, all ovarian epithelial carcinomas, including 31 serous (29 of them showed a papillary pattern), 8 endometrioid, 4 clear cell, 9 poorly differentiated, and 1 mucinous carcinomas. The ovarian cancer cell line IGROV1 was originally derived by Dr. Bernard (Institute Gustave Roussy, Villejuf, France), from a moderately differentiated ovarian carcinoma of an untreated patient; OAW-42 was from Dr. Ulrich U. (Department of Obstetrics and Gynaecology, University of Ulm, Ulm, Germany), whereas OVCAR3, OVCAR8, and SKOV3 were purchased from the American Type Culture Collection. All the cell lines were maintained in RPMI 1640 (Life Technologies), supplemented with 10% (v/v) fetal bovine serum, 3 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin.

miRNA microarray hybridization and quantification. Total RNA isolation was done with Trizol (Invitrogen) according to the manufacturer's instructions. RNA labeling and hybridization on miRNA microarray chips were done as described previously (28) using 5 μg of total RNA from each sample. Hybridization was carried out on our miRNA microarray (Ohio State Comprehensive Cancer Center, version 2.0), which contains probes for 460 mature miRNAs spotted in quadruplicate (235 *Homo sapiens*, 222 *Mus musculus*, and three *Arabidopsis thaliana*) with annotated active sites. Often, more than one probe set exists for a given mature miRNA. Additionally, there are quadruplicate probes corresponding to most precursor miRNAs. Hybridization signals were detected with streptavidin-Alexa Fluor 647 conjugate and scanned images (Axon 4000B) were quantified using the GenePix 6.0 software (Axon Instruments).

Computational analysis of miRNA microarray data. Microarray images were analyzed by using GenePix Pro. Average values of the replicate spots of each miRNA were background subtracted, normalized, and subjected to further analysis. We did a global median normalization of ovary microarray data by using BRB ArrayTools developed by Richard Simon and Amy Peng Lam (29). Absent calls were thresholded to 4.5 before subsequent statistical analysis. This level is the average minimum intensity level detected in the experiments. miRNA nomenclature was according to the Genome Browser[3] and the miRNA database at Sanger Center[4]; in case of discrepancies, the miRNA database was followed. Differentially expressed miRNAs were identified by using the *t* test procedure within significance analysis of microarrays (SAM), a method developed at Stanford University Labs based on recent article of Tusher, Tibshirani, and Chu (30). To identify miRNA signatures, we also applied PAM, which does sample classification from gene expression data, via the "nearest shrunken centroid method" of Tibshirani, Hastie, Narasimhan, and Chu (31).

Northern blotting. Northern blot analysis was done as described previously. RNA samples (10 μg each) were run on 15% polyacrylamide and 7 mol/L urea Criterion precasted gels (Bio-Rad) and transferred onto Hybond-N+ membranes (Amersham). The hybridization was done at 37°C in ULTRAhyb-Oligo hybrization buffer (Ambion) for 16 h. Membranes were washed at 37°C, twice with 2× saline-sodium phosphate-EDTA and 0.5% SDS. The oligonucleotides used as probes were antisense to the sequence of the mature miRNAs (miRNA Registry[5]): miR-200a, 5′-ACATCGTTACCAGACAGTGTTA-3′; miR-141, 5′-CCATCTTTACCAGACAGTGTTA-3′; miR-199a, 5′-GAACAGGTAGTCTGAACACTGGG-3′; miR-125b1, 5′-TCACAAGTTAGGGTCTCAGGGA-3′; miR-145, 5′-AAGGGATTCCTGGGAAAACTGGAC-3′; miR-222, 5′-GAGACCCAGTAGCCAGATGTAGCT-3′; and miR-21, 5′-TCAACATCAGTCTGATAAGCTA-3′.

5S RNA or EtBr gel staining were used to normalize. Two hundred nanograms of each probe were end labeled with 100 μCi [γ-^{32}P]ATP using the polynucleotide kinase (Roche). Blots were stripped in boiling 0.1% SDS for 10 min before rehybridization.

Quantitative real-time PCR. The single tube Taqman miRNA assays were used to detect and quantify mature miRNAs on Applied Biosystems real-time PCR instruments in accordance with manufacturer's instructions (Applied Biosystems). Normalization was done with 18S rRNA. All reverse transcription reactions, including no-template controls and reverse transcriptase minus controls, were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). Gene expression levels were quantified using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Comparative real-time PCR was done in triplicate, including no-template controls. Relative expression was calculated using the comparative C_t method.

Demethylating experiment. OVCAR3 cells were seeded at low density 48 h before treatment with 10 μmol/L 5-aza-2′-deoxycytidine (5-AZA; Sigma). After 24 h of treatment, cells were collected and total RNA was isolated using Trizol reagent. Three replicates for both untreated cells and 5-AZA–treated cells were used to evaluate the miRNA expression by microarray profiling. Differentially expressed miRNAs were identified by using univariate two-class *t* test with random variance model.

Results

A miRNA expression signature discriminates ovarian cancer tissues from normal ovary. We used a custom microarray platform already validated by numerous studies (19) to evaluate miRNA expression profiles on a heterogeneous set of ovarian tissues from different patients. This set included 15 normal ovarian samples, 69 ovarian malignant tumors, and 5 ovarian cancer cell lines, for a total of 89 biologically independent samples. Each tumor sample was derived from a single specimen. Description of sample characteristics is reported in Supplementary Table S1.

The unsupervised hierarchical clustering, based on all the human miRNAs spotted on the chip, generated a tree with a clear

[3] http://genome.ucsc.edu
[4] http://microrna.sanger.ac.uk/
[5] http://www.sanger.ac.uk/Software/Rfam/mirna/

distinction of samples in two main groups, represented by normal tissues and malignant tissues (Fig. 1).

To identify miRNAs differentiating normal versus cancer tissue, we used SAM and PAM tools, and the results obtained from the two types of class prediction analysis were largely overlapping. The SAM comparison between normal and cancer tissues identified 39 miRNAs (with q values <1% and fold changes >3) differentially expressed, 10 up-modulated in tumors and the

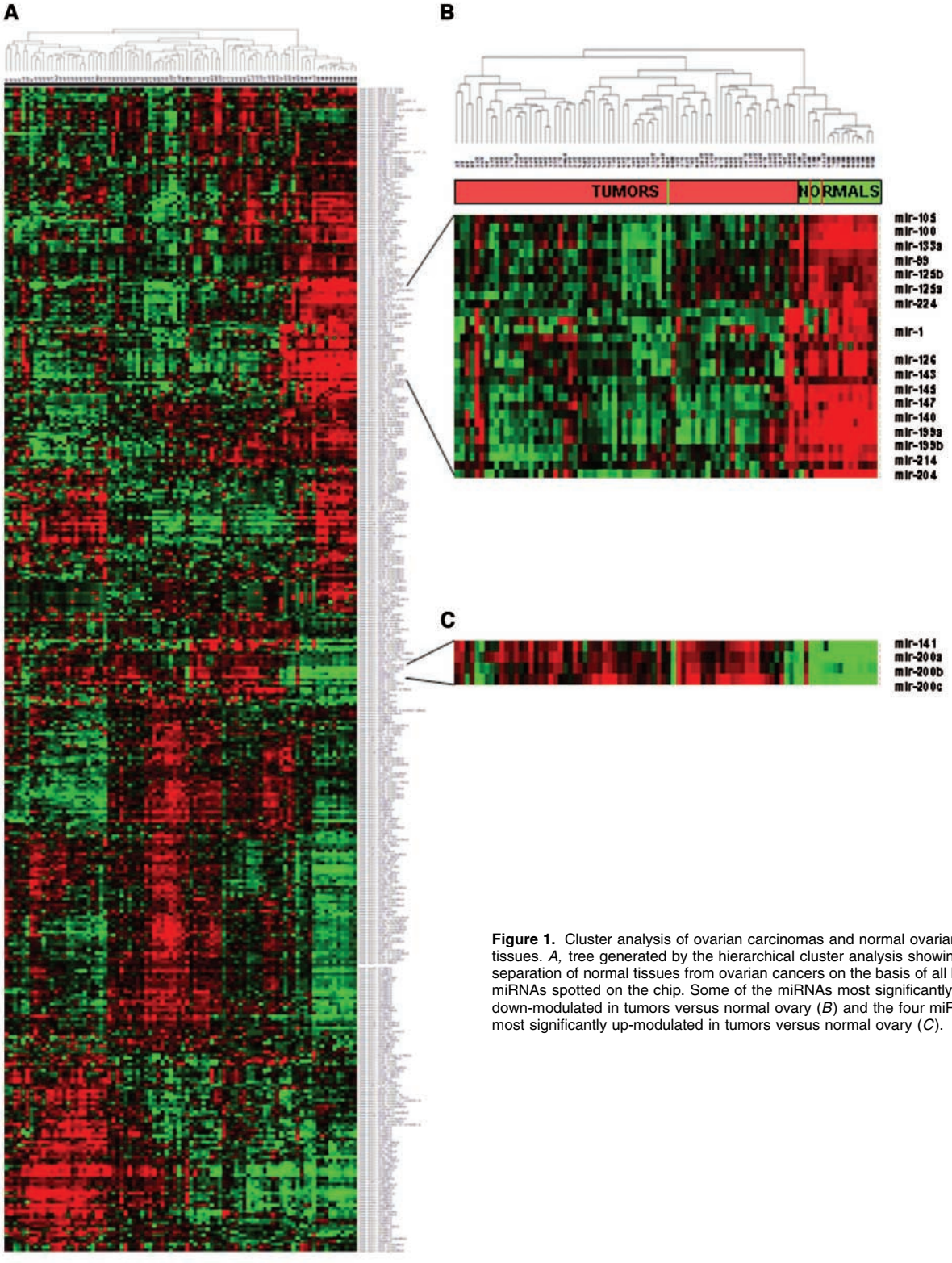

Figure 1. Cluster analysis of ovarian carcinomas and normal ovarian tissues. *A,* tree generated by the hierarchical cluster analysis showing the separation of normal tissues from ovarian cancers on the basis of all human miRNAs spotted on the chip. Some of the miRNAs most significantly down-modulated in tumors versus normal ovary (*B*) and the four miRNAs most significantly up-modulated in tumors versus normal ovary (*C*).

remaining down-modulated (the list is reported in Supplementary Table S2). The PAM analysis in Supplementary Fig. S1 displays the graphical representation of the probabilities (0.0–1.0) of each sample for being a cancer or a normal tissue according to the miRNA signature reported in Table 1, which describes a smaller set of 29 miRNAs, 4 up-modulated (*miR-200a, miR-200b, miR-200c*, and *miR-141*) and 25 down-modulated (being *miR-199a, miR-140, miR-145*, and *miR-125b1* among the most significant) differentiating normal versus tumor with a classification rate of 89%.

Table 1. PAM analysis of miRNAs differentially expressed between tumors and normals

CV confusion matrix (threshold = 3.23866)

True/predicted	Cancer	Normal	Class error rate
Cancer	63	8	0.112676056
Normal	1	14	0.066666667

Misclassification error = 0.11

miRNAs	Cancer score	Normal score
hsa-mir-200c*	0.1152	−0.5454
hsa-mir-200a*	0.1059	−0.5012
hsa-mir-199a †	−0.098	0.4637
hsa-mir-143 ‡	−0.0946	0.4479
hsa-mir-199b ‡	−0.0887	0.4197
hsa-mir-141*	0.0874	−0.4138
hsa-mir-145 ‡	−0.0734	0.3473
hsa-mir-147*	−0.0679	0.3212
hsa-mir-133a ‡	−0.0671	0.3176
hsa-mir-101*	−0.0616	0.2917
hsa-mir-214 †	−0.0607	0.2873
hsa-mir-100 †	−0.0535	0.2533
hsa-mir-140*	−0.0523	0.2474
hsa-mir-126 ‡	−0.0501	0.2371
hsa-mir-224 ‡	−0.0485	0.2294
hsa-mir-9*	−0.0481	0.2277
hsa-mir-105	−0.0461	0.2184
hsa-mir-99a ‡	−0.037	0.1753
hsa-mir-125a ‡	−0.0315	0.1489
hsa-mir-211*	−0.0248	0.1174
hsa-mir-127*	−0.0232	0.11
hsa-mir-200b*	0.0179	−0.0847
hsa-mir-125b-1 †	−0.0177	0.0837
hsa-let-7c ‡	−0.0152	0.0719
hsa-let-7d*	−0.0138	0.0654
hsa-mir-124a*	−0.0121	0.0574
hsa-mir-374	−0.0119	0.0563
hsa-let-7a*	−0.0113	0.0533
hsa-mir-134*	−0.0014	0.0068

NOTE: Of the 39 miRNAs found by SAM analysis, 29 miRNAs, 4 up-modulated and 25 down-modulated, were able to classify normal and tumor samples with a classification rate of 89%. The four miRNAs up-modulated were found amplified in the genomic study done by Zhang et al., 2005; among the miRNAs down-modulated, 10 of 25 were found deleted, 4 are discordant and 11 do not show any copy loss or gain in Zhang study.

*Concordant.

† Discordant.

‡ Unchanged in Zhang study.

To confirm the results obtained by microarray analysis, we carried out Northern blots (Fig. 2*A*) or real-time PCR (Fig. 2*B*) on some of the differentially expressed miRNAs. We analyzed the expression of *miR-200a* and *miR-141*, the most significantly up-modulated in ovarian carcinoma, and the miRNAs most significantly down-modulated, *miR-199a, miR-140, miR-145*, and *miR-125b1*. All the experiments confirmed the results obtained by microarray analysis.

Biopathologic features and miRNA expression. Considering that ovarian epithelial carcinomas occur as different histologic subtypes characterized by distinct morphologic and molecular genetic alterations, we decided to compare the miRNA profile of each of them with the normal tissue to evaluate if miRNA expression profiles are different in distinct histotypes of ovarian carcinomas. Complete lists resulting from SAM analyses are reported in Supplementary Table S3, whereas a summary is shown in the Venn diagram in Fig. 3: two of four miRNAs most significantly up-modulated (Fig. 3*A*) in tumors versus normal tissue, *miR-200a* and *miR-200c*, are up-modulated in all the three histotypes considered (serous, endometrioid, and clear cell), whereas *miR-200b* and *miR-141* up-modulation is shared by endometrioid and serous histotypes. Moreover, the endometrioid histotype shows the up-modulation of three additional miRNAs, *miR-21, miR-203*, and *miR-205*. Nineteen miRNAs, including *miR-125b1, miR199a*, and *miR-140*, are down-modulated (Fig. 3*B*) in all the three histotypes examined in comparison with normal tissue, whereas four are shared in each paired analysis of the different signatures: *miR-145*, for example, is down-modulated in both serous and clear cell carcinomas; *miR-222* is down-modulated in both endometrioid and clear cell carcinomas.

Considering the tumors classified as "mixed" and "poorly differentiated," we found that the first group revealed a signature with characteristics of different histotypes, sharing for example the overexpression of *miR-200c* and *miR-181* with the endometrioid carcinomas and the down-modulation of *miR-214* with the serous, whereas the "poorly differentiated" tumors have a quite different pattern of miRNA expression (Supplementary Table S3).

We then compared miRNA expression of different groups of tumors paired as reported in Supplementary Table S4, and in particular, we compared the two most numerous histotypes, serous and endometrioid. When considering the miRNAs differentially expressed in endometrioid carcinomas compared with serous, we found *miR-212* up-modulated and *miR-302b** and *miR-222* ($P < 0.05$, t test analysis of microarray data in Fig. 4*A*) among the miRNAs most significantly down-modulated. In Fig. 4*B*, a Northern blot on a small set of samples verifies *miR-222* overexpression in serous tumors compared with endometrioid.

We then focused our attention on other clinicopathologic features associated with tumor specimens: whereas no miRNAs were found significantly differentially expressed in relation to the age of patients, other tumor characteristics seemed to affect miRNA expression, such as lymphovascular invasion and ovarian surface, tubal, uterus, and pelvic peritoneum involvement (Supplementary Table S5).

To investigate if there were miRNAs associated with different grade or stage of the disease, we did comparative analyses considering all the tumors or only the serous histotype, which was the most numerous, but we did not obtain any significant miRNA differentially expressed.

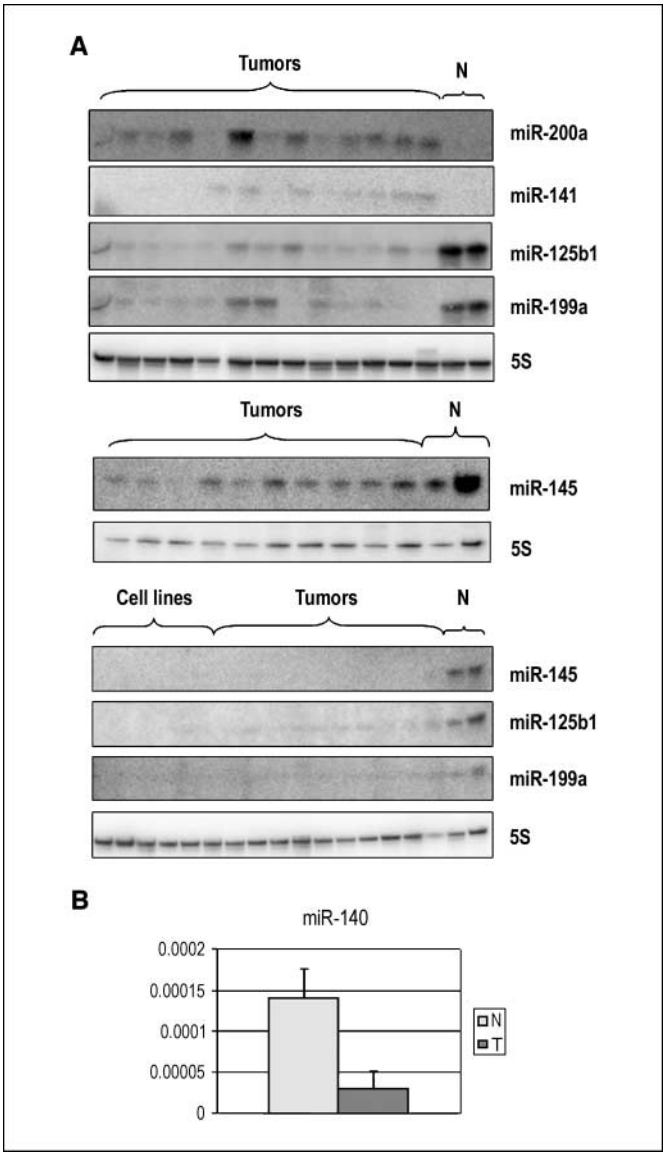

Figure 2. *A,* Northern blot analysis of human ovarian carcinomas with probes of miR-200a, miR-141, mir-199a, mir-125b1, and miR-145. Evaluation of miR-199a, miR-125b1, and miR-145 on human ovarian cell lines. The 5S probe was used for normalization of expression levels in the different lanes. *B,* real-time PCR was used to verify the miR-140 down-modulation in tumors compared with normal samples.

Confirmed and potential targets for miRNA members of various signatures. Using the DianaTarbase,[6] we looked for confirmed targets of some of the most significant miRNAs resulting from our analyses, finding some interesting data: *ERBB2* and *ERBB3* receptors, for example, are targeted by *miR-125* (32); *miR-101*, down-modulated in ovarian carcinoma, has been shown targeting the oncogene *MYCN* (33). We then analyzed their potential targets using the miRGen database[7] and evaluated for some of these molecules the expression levels in ovarian carcinoma: all the four most significantly up-modulated miRNAs, *miR-200a, miR-200b, miR-200c*, and *miR-141*, for example, have as common putative target the oncosuppressor BAP1, BRCA1-associated protein, down-modulated in ovarian cancer. The information obtained is summarized in Supplementary Table S6.

Epigenetic regulation of miRNA expression. To evaluate if an aberrant DNA methylation pattern could also contribute to the altered miRNA expression characterizing the human ovarian carcinoma, we analyzed the miRNA profiling of the ovarian cell line OVCAR3, before and after treatment with the demethylating agent 5-AZA. The analysis of microarray data showed 11 human miRNAs differentially expressed, 9 up-modulated and 2 down-modulated ($P < 0.001$, significance threshold of each univariate test), being *miR-21, miR-203, miR-146b, miR-205, miR-30-5p*, and *miR-30c* the most significantly induced on treatment (the miRNAs differentially expressed are listed in Fig. 5*A*, whereas the resulting hierarchical cluster tree is reported in Fig. 5*B*). Real-time PCRs to verify the up-modulation of the five most significantly induced miRNAs are described in Fig. 5 as graphical representation of miRNA expression levels (Fig. 5*C*), and *miR-21* was also validated by Northern blot (Fig. 5*D*).

Interestingly, *miR-21, miR-203*, and *miR-205* are overexpressed in ovarian carcinomas compared with normal tissues (see SAM analysis in Supplementary Table S2 and Venn Diagram in Fig. 3): the reactivation of these miRNA genes after demethylating treatment suggests that the hypomethylation could be the mechanism responsible for their overexpression *in vivo*. We confirmed the overexpression of *miR-21*, the most significant miRNA induced on treatment, doing a Northern blotting (Supplementary Fig. S2*A*) on a panel of human ovarian carcinomas and two normal tissues. Moreover, using the CpG Island Searcher Program (34), we verified that *miR-21* and *miR-203* are associated with CpG islands, being the *miR-203* embedded in a CpG island 875 bp long and the *miR-21* characterized by a CpG island ≈2 kb upstream the mature sequence (Supplementary Fig. S2*B*), whereas *miR-205* does not show any CpG island in a region spanning 2 kb upstream its mature form.

Discussion

In this study, we show that miRNAs are aberrantly expressed in human ovarian cancer. The overall miRNA expression could clearly separate normal versus cancer tissues, identifying a number of miRNAs altered in human ovarian cancer and probably involved in the development of this neoplasia.

The expression of all the four miRNAs we found most significantly up-modulated, *miR-200a* and *miR-141*, belonging to the same family; *miR-200b* (localized in the same region of *miR-200a,* at chromosome 1p36.33); and *miR-200c* (localized in the same region of *miR-141,* at chromosome 12p13.31), is concordant with the results obtained at genomic level by Zhang et al. (24), suggesting that the mechanism driving their up-modulation could be the amplification of the miRNA genes. Interestingly, all these miRNAs have a common putative target: the oncosuppressor BAP1, BRCA1-associated protein (24). The altered expression of GATA factors, found and proposed as the underlying mechanism for dedifferentiation in ovarian carcinogenesis (35), may also be driven by miRNA deregulation. In particular, GATA6, lost or excluded from the nucleus in 85% of ovarian tumors, may be regulated by *miR-200a*, and GATA4, absent in the majority of ovarian cancer cell lines, may be targeted by *miR-200b* (Supplementary Table S5).

Among the down-modulated genes, notably, we found *miR-125b1*, altered also in breast cancer, as well as *miR-145* (18); *mir-199a*,

[6] http://www.diana.pcbi.upenn.edu/tarbase.html
[7] http://www.diana.pcbi.upenn.edu/miRGen.html

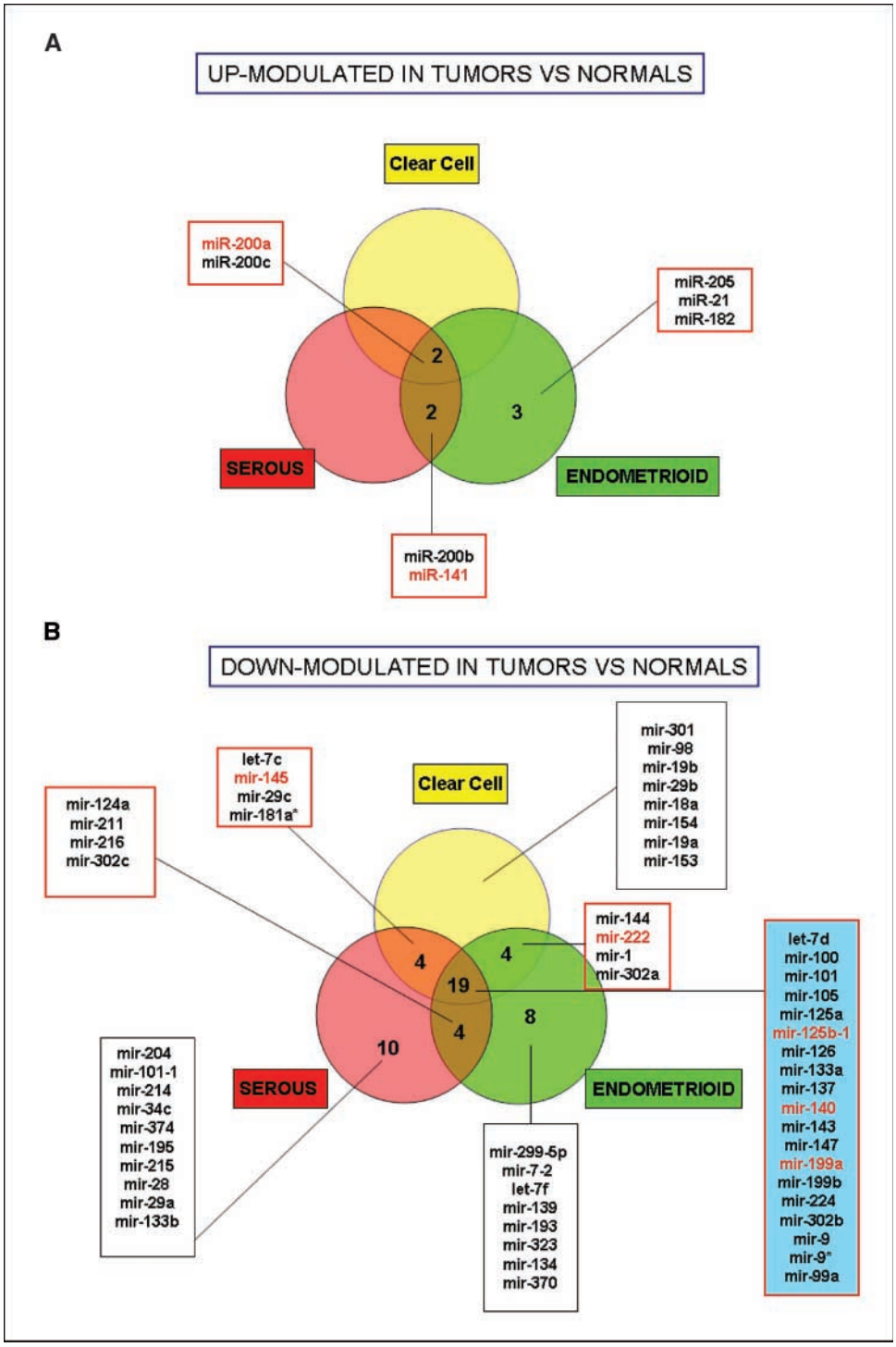

Figure 3. Venn diagram showing the miRNA signatures characterizing different ovarian carcinoma histotypes (serous, endometrioid, and clear cell) compared with the normal tissue (*A*, miRNAs up-modulated; *B*, miRNAs down-modulated).

recently shown down-modulated in other tumors, as hepatocellular carcinoma (36); and *miR-140*, deleted in ovarian carcinoma (24). Interestingly, *miR-140* is indeed located at chromosome 6q22, a fragile region often deleted in ovarian tumor, and it is predicted to target important molecules as c-SRK, MMP13, and FGF2.

Even if the normal control available in this study is represented by whole normal ovary, our data could identify a number of miRNAs altered in human ovarian carcinoma and probably involved in the biology of this malignancy.

In fact, the miRNA signatures obtained comparing different histotypes of ovarian carcinomas (serous, endometrioid, clear cell, and mixed) with the normal tissue are overlapping in most cases, but they also reveal a number of miRNAs that seem to be "histotype specific": the endometrioid tumors, for example, share with the others the four most significantly up-modulated miRNAs (*miR-200a, miR-200b, miR-200c,* and *miR-141*) but also present overexpression of *miR-21*, known to be misregulated in numerous solid tumors (18, 37, 38) and to exert an antiapoptotic role in different

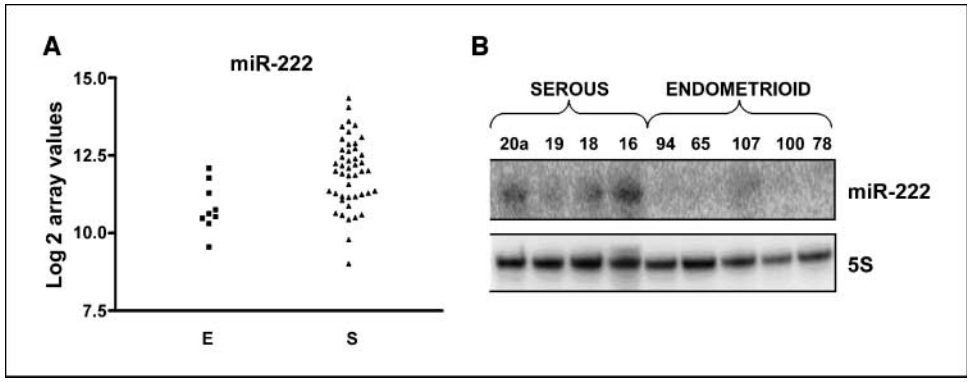

Figure 4. *t* test graphic representation of miR-222 microarray data expression in serous (*S*) and endometrioid (*E*) tumors (*A*) and verification by Northern blot on a smallest set of samples (*B*).

cellular systems (39, 40), *miR-205* and *miR-182*. Endometrioid tumors also present down-modulation of several miRNAs in comparison with the other classes of tumors, for example, *miR-222*, already shown targeting c-Kit (41), being involved in cancer (42–44) and down-modulated under folate-deficient conditions (45).

These differences enforce the fact that different histotypes represent biologically and pathogenetically distinct entities of EOCs, although they are currently treated with identical therapeutic strategies. Microarray analysis has confirmed recently that different histotypes (serous, mucinous, endometrioid, and clear cell) show the alteration of different pathways, probably reflecting the gene expression pattern of the organ of origin (respectively fallopian tubes, colonic mucosa, and endometrium; ref. 46).

Notably, many of the miRNAs differentially expressed are predicted to target molecules involved in pathways differentially activated depending on the histotype. *miR-212*, for example, down-modulated in serous carcinoma, has as putative target WT1, overexpressed in this subgroup of ovarian carcinomas (47). Another putative target of *miR-212* is BRCA1: mutated in

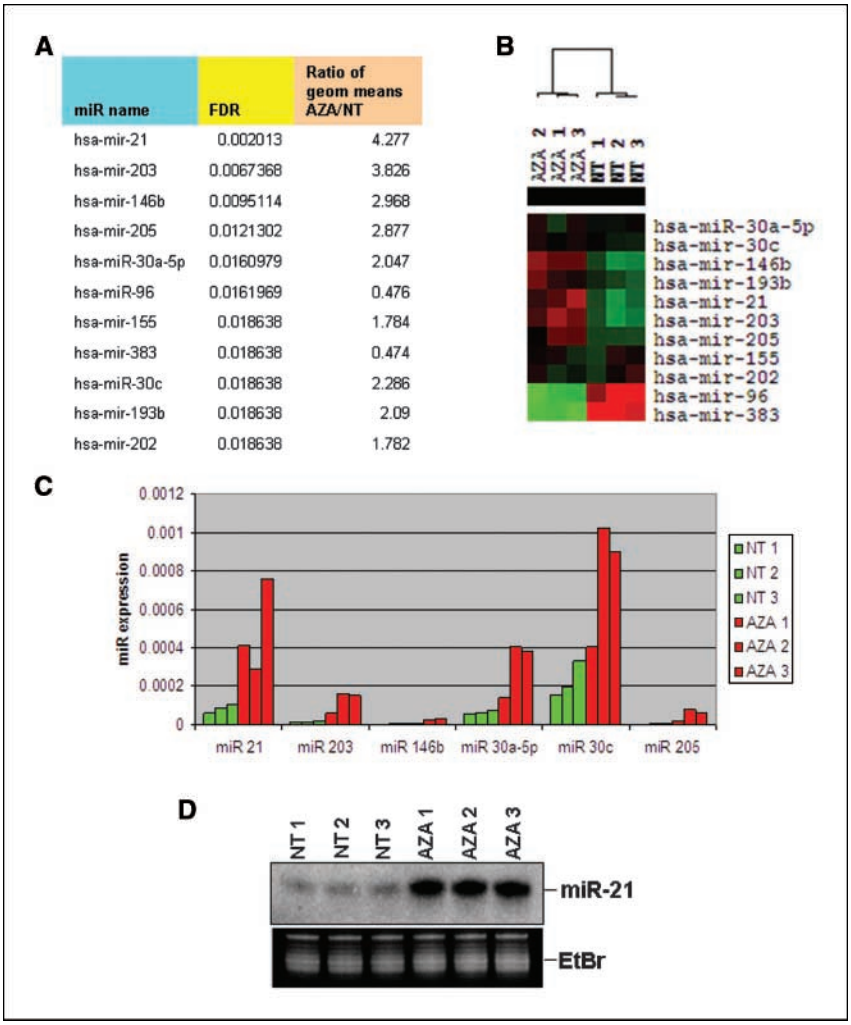

Figure 5. Expression pattern of miRNAs in OVCAR3 cell line before and after treatment with the demethylating agent 5-AZA. *A,* table reporting the most significant miRNAs differentially expressed resulting from the microarray profiling. *B,* hierarchical cluster tree representation. *C,* real-time PCR to verify the up-modulation of the five most significantly induced miRNAs, reported as graphical representation of miRNA expression levels. *Columns,* independent experiment resulting from the average of three technical replicates. *D,* Northern blot showing the up-modulation of miR-21 after treatment, normalized with EtBr gel staining.

hereditary ovarian cancer, this molecule has been found recently involved also in the pathoetiology of sporadic OEC, where a loss of gene function due to epigenetic alterations has been observed more commonly (48). The decreased BRCA1 expression could be determined by overexpression of one or more miRNAs.

miR-299-5p and *miR-135b*, up-modulated in serous histotype compared with endometrioid, are supposed to target, respectively, Delta-like 1 (DLK1) and msh homeobox 2 (MSX2), overexpressed in endometrioid carcinomas (47). Compared with the other tumors, clear cell carcinomas show expression levels of *miR-30-5p* and of *miR-20a* opposite (46) to two putative targets, retinol binding protein 4 (RBP4) and solute carrier 40-iron-regulated transporter, member 1 (SLC40A1), respectively. Compared with the normal tissue, clear cell carcinoma also shows lower expression of *miR-18a, miR-19a*, and *miR-19b*, suggesting a possible down-modulation of the *cluster 17-92* (already validated as deleted by Zhang et al.). This cluster, involved in the intricate regulation mediated by E2F1 and c-Myc, seems to have a duplex nature of putative oncogene, as suggested recently in B-cell lymphoma (15), or tumor suppressor: in hepatocellular carcinoma, for example, loss of heterozygosity at the locus coding the *miR-17-92 cluster* (13q31) has been reported (49). In ovarian carcinoma, at least in clear cell histotype, it could also exert a role of oncosuppressor. Our data suggest indeed that miRNAs may have a regulatory role in the process of differentiation leading to the development of a specific subtype of EOC. Interestingly, poorly differentiated carcinomas have a quite different pattern of miRNA expression, showing up-modulation of several miRNAs in comparison with normal ovary. More intriguingly, one of them, *miR-373*, has been described recently as putative oncogene in testicular germ cell tumors (16).

The absence of miRNAs significantly differentially expressed in relation to tumor stage or grade might be explained by the fact that our set of samples is mostly represented by advanced stage tumors, as expected considering the late diagnosis of this kind of neoplasia; however, the difference in size among the different groups of samples could have represented a limit for the statistical analysis. Alternatively, miRNAs might be important for the development of human ovarian carcinoma but not for the progression of the disease.

Resulting from our analyses, a number of miRNAs overexpressed but not reported as amplified in Zhang study, as well as down-modulated but not deleted, the involvement of an epigenetic regulatory mechanism could actually exert a role on miRNA expression in human EOC. Indeed, among the most significant miRNAs induced after demethylating treatment of an ovarian cell line, we found *miR-21, miR-203*, and *miR-205* up-modulated in ovarian cancer. Moreover, *miR-203* and *miR-21* are associated with a CpG island (miR-203 is embedded in a CpG island, whereas *miR-21* has a CpG island $\approx$2 kb upstream its mature sequence), supporting the idea that the demethylation leads to the reactivation of these miRNA genes. Notably, *miR-21* has already been described up-modulated in several human tumors and having an antiapoptotic role in different cellular models. These data suggest that the DNA hypomethylation could be an epigenetic mechanism responsible for the *in vivo* overexpression of potentially oncogenic miRNAs.

To the best of our knowledge, this is the first report describing a complete miRNA expression profiling in human EOCs, focused on the identification of miRNAs differentially expressed in carcinomas versus normal ovary and in different subgroups of tumors. Our data suggest the important role that miRNAs can exert on the pathogenesis and on the development of different histotypes of ovarian carcinoma and identify altered DNA methylation as a possible epigenetic mechanism responsible for the aberrant expression of miRNAs not affected by genomic changes.

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