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,7 Stefano Volinia,2 Chang-Gong Liu2, Hansjüerg Alder,2 George A. Calin,2 Sylvie Ménard,1 and Carlo M. Croce2

1Molecular Biology Unit, Department of Experimental Oncology, Fondazione Istituti di Ricovero e Cura a Carattere Scientifico, Istituto Nazionale Tumori, Milano, Italy and 2Department 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 micro-RNAs (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 oncopgenes 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...
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 polycistrion 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 clinicopathologic 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 aberrant miRNA expression, identifying the presence of genomic

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 inadequately differentiated, and 1 mucinous carcinomas. The ovarian cancer cell line IGROV1 was originally derived by Dr. Bernard (Institute Gustave Roussy, Villejuif, 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

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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


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%.

To confirm the results obtained by microarray analysis, we carried out Northern blots (Fig. 2A) or real-time PCR (Fig. 2B) 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. 3A) 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. 3B) 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. 4A) among the miRNAs most significantly down-modulated. In Fig. 4B, 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 clinopathologic 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.

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

<table>
<thead>
<tr>
<th>CV confusion matrix (threshold = 3.23866)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>True/predicted</strong></td>
</tr>
<tr>
<td>Cancer</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Misclassification error = 0.11</td>
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<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Cancer score</th>
<th>Normal score</th>
</tr>
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<tbody>
<tr>
<td>hsa-mir-200c*</td>
<td>0.1152</td>
<td>-0.5454</td>
</tr>
<tr>
<td>hsa-mir-200a*</td>
<td>0.1059</td>
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<td>hsa-mir-199a†</td>
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<tr>
<td>hsa-mir-143‡</td>
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<td>0.4479</td>
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<td>hsa-mir-199b‡</td>
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<td>hsa-mir-133a‡</td>
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<tr>
<td>hsa-mir-134*</td>
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<td>0.0068</td>
</tr>
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</table>

* Concordant.
† Discordant.
‡ Unchanged in Zhang study.
miR-200a, miR-200b, miR-200c, some of these molecules the expression levels in ovarian receptors, for example, are targeted by ERBB3 potential targets using the miRGen database and evaluated for targeting the oncogene MYCN. From our analyses, finding some interesting data: confirmed targets of some of the most significant miRNAs resulting with normal samples. PCR was used to verify the miR-140 down-modulation in tumors compared with normal tissues. Moreover, using the CpG Island Searcher Program (34), we verified that 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. 5A, whereas the resulting hierarchical cluster tree is reported in Fig. 5B). 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. 5C), and miR-21 was also validated by Northern blot (Fig. 5D).

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. S2A) 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. S2B), 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,
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
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...
hereditary ovarian cancer, this molecule has been found recently involved also in the pathobiology 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 (DLLK1) 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 (BBP4) 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 hepato-cellular 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 ≈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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References


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