Title: miRNA landscape in Stage I Epithelial Ovarian Cancer defines the histotype specificities

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STATEMENT OF TRANSLATIONAL RELEVANCE

The present study shows that different histotypes of stage I ovarian cancer exhibit divergent miRNA profiles, thus supporting the hypothesis that ovarian tumors with different histotypes are biologically distinct and thus they should be treated as different neoplastic diseases.

In particular, the pattern of expression of some miRNAs was found specific for clear cell or for mucinous ovarian carcinomas, a finding of potential diagnostic and therapeutic importance.
ABSTRACT

**Purpose:** epithelial Ovarian cancer (EOC) is one of the most lethal gynecological diseases, with survival rate virtually unchanged for the past 30 years. EOC comprises different histotypes with molecular and clinical heterogeneity, but up till now the present gold standard platinum-based treatment has been performed without any patient stratification. The aim of the present study is to generate miRNA profiles characteristic of each stage I EOC histotype, in order to identify subtype-specific biomarkers to improve our understanding underlying the tumor mechanisms.

**Experimental Design:** a collection of 257 snap-frozen stage I EOC tumor biopsies was gathered together from three tumor tissue collections and stratified into independent training (n=183) and validation sets (n=74). Microarray and qRT-PCR were used to generate and validate the histotype-specific markers. A novel dedicated resampling inferential strategy was developed and applied to identify the highest reproducible results. mRNA and miRNA profiles were integrated to identify novel regulatory circuits.

**Results:** robust miRNA markers for clear cell and mucinous histotypes were found. Specifically, the clear cell histotype is characterized by a five-fold (log scale) higher expression of miR-30a and miR-30a*, while mucinous histotype has five-fold (log scale) higher levels of miR-192/194. Furthermore a mucinous-specific regulatory loop involving miR-192/194 cluster and a differential regulation of E2F3 in clear cell histotype were identified.

**Conclusions:** our findings demonstrated that stage I EOC histotypes have their own characteristic miRNA expression and specific regulatory circuits.
INTRODUCTION

Despite the increasing molecular knowledge of tumor biology which underpins the development of new therapeutic and clinical management strategies, EOC is one of the most lethal gynecological diseases, with a five-years survival rate virtually unchanged for the past 30 years (1).

Notwithstanding the enormous effort, the causes of its pathogenesis are still unknown as well as the mechanism of disease in the early phases of the carcinogenesis. Most EOC studies have been performed in patients characterized by stage III-IV, the most frequent clinical manifestation, while the low incidence of stage I EOCs has hampered the possibility to collect sizable cohorts of tumor biopsies and to perform stage I dedicated studies.

The importance of a better knowledge of stage I EOC is particularly made evident by the recent proposal regarding the EOC origin described by Kurman and Shih (1). Based on this theory, all histological types, of what we currently define "ovarian carcinoma", originate from different organs sharing the same anatomic location.

Low-grade serous, mucinous, endometrioid and clear cell histotypes represent the great majority of type I EOCs, which usually have a slow progression rate, are generally confined to the ovary, lack of p53 mutations and show a lineage with the corresponding benign neoplasm through an intermediate step called borderline tumor stage (1). This notion is not universally accepted, however there is a general consensus on the heterogeneous molecular and clinical characteristics of the different EOC histotypes (2).

Köbel et al, in a retrospective study, tested 21 candidate markers in a cohort of 500 advanced stages EOCs, demonstrating that the association between biomarker expressions and survival rates varies among subtypes. The results suggest that the different histological types are indeed distinct diseases, intimating that including different histotypes in a single cohort may not only confound survival analyses, but also it can lead to erroneous conclusions (3).

The role of microRNAs in high-grade EOCs has been studied in the past, highlighting a strong involvement of both the non-coding RNAs and the enzymes composing the miRNA processing
machinery (4,5). Data previously published by our group deciphered a molecular circuit in stage I EOC, revealing miR-200c as an independent prognostic factor (6,7). Focusing our study on the microRNA profiles, we studied the differences among stage I EOC histological types looking for specific markers and regulatory circuits. The histotype specificities are key knowledge to understand the treatment efficacies and to guide the development of new therapies. At the same time, a better characterization of early EOCs can be potentially helpful to deeply clarify the mechanism of the disease.

Here, we present the first retrospective study focused on histotypes, exclusively based on stage I EOC samples, including a collection of 257 snap frozen tumor biopsies gathered by three independent tumor tissue banks.
MATERIALS AND METHODS

Tissue sample collection. A cohort of 257 snap frozen tumor biopsies was gathered together from three independent snap frozen tumor tissue collections (Supplementary Section S1). Tumor tissue samples, collected at the time of surgery, were sharp-dissected and snap frozen in liquid nitrogen within 15 min from resection, and then stored at -80°C. Patients underwent a complete staging procedure, according to the International Federation of Gynecological and Obstetrics criteria (FIGO) (8). All biopsies selected for the study belonged to patients naive to chemotherapy and with diagnosis of stage I EOC. All patients with a suspected mucinous ovarian cancer underwent preoperative colonoscopy and gastroscopy, in order to exclude metastatic disease from a gastrointestinal tumor. Even when the diagnosis of mucinous histology was made postoperatively, patients underwent endoscopic examinations within one month from surgery. Patients who were found to have ovarian metastases from gastrointestinal tumors were not considered for the present study.

Clinical and anatomo-pathological patient information was registered, and follow-up data were obtained from periodic gynecological and oncological check-up. Clinical data were analyzed following the procedures described in Supplementary Section S2. The tumor content of the specimens was assessed by haematoxylin and eosin staining to check epithelial purity. While malignant cells content in borderline tumors ranged from 45% to 70%, in malignant tumors only specimens containing more than 70% of epithelial tumor cells were used. Tumor grade and histological type were determined following World Health Organization (WHO) standards. A written informed consent was obtained from all the patients enrolled in the study, which has been performed following the Declaration of Helsinki set of principles. The local scientific ethical committees of all the centres participating to the study approved the collection and the use of tumor samples. HOSE primary cell lines were established after sterile processing of samples from surgical biopsies, as described in Supplementary Section S3.

Expression experiments and analyses. miRNA extraction, labeling and hybridization were performed as previously published (7) and more details are described in Supplementary Section S4. miRNA profiles raw data have been submitted to ArrayExpress (E-MTAB-1067). A resampling strategy
methodology was applied as described in Supplementary Section S5. Gene expression dataset (B1 collection; GSE8841-2 (6)) was treated following the Supplementary Section S6 procedure and MAGIA2 software (9,10) was used to perform the integrative analysis.

**Validation using qRT-PCR.** miRNA and gene expression levels were validated by qRT-PCR using Sybr Green protocol (Qiagen, Milano Italy) on an Applied Biosystems 7900HT instrument. Experiments were run in triplicate, using 384-well reaction plates in an automatic liquid handling station (epMotion 5075LH; Eppendorf, Milano, Italy). Analysis was performed as previously described (7), using four independent housekeeping genes as listed in Supplementary Section S7. Two-sided Student's t-test for training set and Wilcoxon test for validation set were used to verify among groups mean differences. p-value < 0.05 were considered statistically significant.

**Luciferase assays.** pmirGLO-CUL4 (pCUL4), pmirGLO-PSME3 (pPSME3) and psiCHECK-BMI1 (pBMI1) plasmids, containing predicted miRNA binding sites for miR-194, were PCR sub-cloned into the pmirGLO or psiCHECK-2 luciferase reporter plasmid using primers pair sequences reported in Supplementary Section S7, as follows: 95°C for 1 min (1 cycle); 95°C for 30 sec, annealing temperature (Ta) for 1 min, 68°C for 2 min (40 cycles), and a final extension step at 68°C for 1 min (see for different Ta, Supplementary Table S7.1). Human embryonic kidney (HEK) 293 cells, originally obtained from American Type Culture Collection (ATCC), routinely grown at 37°C, 5% CO₂ in DMEM medium (Life Technologies, Milano Italy), supplemented with 10% fetal bovine serum (Lonza, Milano, Italy) and 1% L-Glutamine 200mM (Biowest, Milano, Italy) were used for luciferase assay. HEK 293 cells were tested and authenticated by short tandem repeat profile using the Cell ID System (Promega) and ABI Prism 310 Genetic Analyzer (Applied Biosystems), before last freezing (November 2012).

Sub-confluent HEK 293 cells, seeded in 96-well reaction plates, were co-transfected with plasmids (10 ng) and with 2 pmol of synthesized, double-stranded miRNA-194 (or siRNA scramble (Qiagen) using Lipofectamine 2000 (Life Technologies). 24 hours after transfection, firefly and renilla luciferase activity was measured consecutively in a microplate reader (Tecan Infinite®M200, Milano, Italy).
using the Dual Luciferase Assay System (Promega, Milano, Italy). Co-transfection of psiCHECK-ZEB1 (pZEB1, kindly provided by dr. Goodall) with synthetic miR-200c (Qiagen) was used as positive control. Empty vectors were transfected as negative controls. Each sample was assayed in six replicates and repeated twice.

RESULTS

Patient characteristics

To identify a miRNA signature able to characterize EOC histotypes, we analyzed a collection of 257 snap frozen tumor biopsies obtained from primary surgery on stage I EOC patients naive to chemotherapy, gathered together from three tumor tissue collections hereafter called A, B and C (Supplementary Section S1).

Given that borderline ovarian tumors are histologically characterized as epithelial tumors, they are included in the study, even if they are defined as low malignant potential tumors. Borderline patients, mainly with serous and mucinous histology, represented the ~20% of the entire patient cohort (38 patients in the training set, 15 patients in the validation set). Aware that the borderline inclusion criteria could be considered controversial, all the analyses reported in this study were performed including and excluding borderline patients, with overlapping results.

Median patient ages at diagnosis in the three collections were similar, as well as the distribution among histotypes and grades of nuclear differentiation.

As previously reported in different early-stage EOC dedicated studies (11–13), both univariate and multivariate analyses did not reveal any difference in survival rates among different histotypes. The grade of tumor was the most significant prognostic feature: increasing grade at the time of diagnosis was associated to decrease in patient survival (Supplementary Section S2). This result confirmed that our cohort of patients is consistent with data reported in the literature for stage I EOC and that is both representative of clinical settings and suitable for downstream analyses.
A schematic representation of the experimental and computational strategies adopted in this study is reported in the flow chart of Fig. 1. The entire cohort of patients was subdivided into a training set (n=183; collection A, B1 and B2) and a validation set (n=74; collection B3 and C). The training set was used to: i) generate miRNA expression profiles and define marker identification procedure (A+B1+B2); ii) integrate miRNA profiles with gene expression patterns (6) in a subset of patients (B1), and iii) validate gene and miRNA expression by qRT-PCR (A+B1+B2). The validation set was used only to independently validate the expression by qRT-PCR.

Analysis of microRNA landscape

To measure the miRNA expression in a high-throughput manner, training set biopsies (n=183) were profiled using microarray. Fig. 2A shows the heat-map of the 250 miRNA values obtained after the pre-processing and data normalization. On a global scale, a large part of the entire set of miRNAs was similar across samples and it does not help to separate patients by histotype and grade. Then, the expression levels have been analyzed and Supplementary Section S4 shows the lists of significantly deregulated miRNAs for each subtype comparison, including and excluding borderline samples. It is noteworthy that, among all the different comparisons, the mucinous subtype showed the highest number of differentially expressed features when compared with the other subtypes (Fig. 2B). If the number of differentially expressed miRNAs is a measure of the differences among histological types, our results support the hypothesis that the mucinous subtype is markedly different from all the others. Although all the miRNAs reported above have significant adjusted p-value, due to patient variability among subtypes, only few of them can be exploited as subtype-specific markers, which are miRNAs that almost perfectly predict the histotype of independent samples. Then, with the aim to identify miRNA markers among those differentially expressed, we performed a dedicated resampling strategy (Supplementary Section S5). A resampling score was assigned to each miRNA, following the rule that the higher the score, the higher the prediction power. Only ten miRNAs were found commonly deregulated across all possible comparisons of subtypes, and only three miRNAs reached the
maximum score: miR-192 and miR-194 were highly expressed in the mucinous subtype, and miR-30a was highly expressed in clear cell EOCs. Otherwise, no miRNA could be identified as potential marker for endometrioid or serous histotypes. Thus, we reasoned that miR-192/194 and miR-30a could be considered EOC histotype-specific markers. Although characterized by a high resampling score, miR-30a* did not reach the RS maximum in all comparisons. However, due to its physical association with miR-30a, it was included in the list of miRNAs selected for validations. It is to note that, repeating the analyses excluding the borderline samples, the results obtained were similar (Table 1 and Supplementary Section S4). In Fig. 2C, we reported the distribution of miRNA expression levels of miR-192/194 and miR-30a/30a* in the four histotypes. Using these expression profiles to re-cluster the entire set of patients, we obtained the cluster in Fig. 2D. The classification of subtypes improved dramatically, separating mucinous and clear cell histotypes from the others, while endometrioid and serous sample subtypes, as expected, generated a single and heterogeneous cluster. Interestingly, the mucinous cluster in Fig. 2D was preferentially characterized by the presence of low-grade patients (grade 1 and borderline). Given this evidence, we investigated the presence of potential variability in expression level across grades within subtypes. We found that, although not significant, miR-192/194 expression levels slowly decreased with the increasing grade and, interestingly, this decrease was associated with reduced survival (Supplementary Section S8). Taken together, our results show that, independently of inclusion criteria (i.e. considering or excluding borderline tumors), miR-192/194 and miR-30a/30a* are histotype-specific markers for stage I EOC.

Moreover, in order to assess whether miR-192/194 and miR-30a/30a* were tumor specific markers, we compared the miRNA expression of EOC histotypes with those of HOSE (Human Ovarian Surface Epithelium) cells, derived from normal ovarian epithelial tissue of patients undergoing surgery for benign pathologies (Supplementary material S3). We performed expression measurements using both microarray and qRT-PCR.

Data reported in Supplementary Fig. S3.1 show that the number of miRNAs, found differentially expressed between HOSE and the EOC histotypes, was higher than the number reported for the
comparison within the tumor histotypes. miR-192/194 and miR30a/30a* data, obtained using array (Supplementary Table S3.1) and qRT-PCR validations (Supplementary Table S3.2), indicated that the previously reported markers were not expressed in HOSE cells.

Relationship between miRNA and gene expression

To depict more in detail the molecular circuits behind each histotype, we focused on functional miRNA-target relationships by approaching an in silico integration between miRNAs and gene expression profiles. A portion (B1, n=76) of the patient cohort was previously used for gene expression analysis (GSE8841-2), and their clinical features are reported in Supplementary Section S6 (6). Anti-correlated expressions were searched between miRNA and their putative target genes. We found miR-192/194 and miR-30a among the top results, confirming the prominent role of these miRNAs (Supplementary Section S6). Then, for downstream analyses, we chose the top significant and interesting anti-correlated targets of the four miRNA markers: *BMI1, CIAO1, PSME3, CUL4A, MAPK4A, UBEV2V2, APOLIA, PTGER3, E2F3, ZNF64* (Supplementary Table S6.2).

Validation using qRT-PCR

We measured by qRT-PCR the expression levels of miRNA markers and their selected putative targets in the training set and in an independent validation set (Fig. 1). To avoid potential errors due to batch effects, new batches of snap frozen material for the entire cohort of samples were used. All the analyses were performed including and excluding borderline patients, leading to the same results (Table 2 and Supplementary Section S7).

Within the training set, data show that miR-192/194 were, roughly in log scale, five to eight folds over-expressed in mucinous compared to the other histotypes and miR-30a/30a* mirror the same trend, being in log scale five to seven folds over-expressed in the clear cell compared to the other histotypes (p<0.001). Seven out of the selected ten putative target genes were confirmed by qRT-PCR (Supplementary Section S7).
In the validation set, miRNA markers (p<0.005) and six of the seven putative target genes (p<0.05) previously confirmed in the training set, were re-confirmed. In particular BMI1, CIAO1, PSME3, CUL4A, PTGER3 were three to four folds (log scale) down-regulated in the mucinous compared to the other subtypes, and E2F3 was approximately four folds (log scale) down regulated in the clear cell compared to the other histotypes. For reason of completeness, also MDM2 and CDKN2A were included in the list of analyzed genes, because they belong to the same circuit. Box-plots in Fig. 3A summarize the qRT-PCR expression values of all the genes in the circuit, analyzed in all 257 patients enrolled in the study. BMI1, PSME3, CUL4A, miR-192/194 showed expression concordant to the previous analyses, MDM2 was three to four folds in log scale down-regulated and CDKN2A was three to five fold in log scale up-regulated in the mucinous histotype (Supplementary Table S7.6).

Functional binding assays: downstream target analysis

Among the genes of the circuit previously validated, not only BMI1, a known target of miR-194 (14), but also CUL4A and PSME3 could be novel and attractive candidate targets. Luciferase reporter vectors containing the entire CUL4A, PSME3 and BMI1 3’UTR, with highly conserved miR-194 binding motif, were constructed and luciferase activity was assayed. Results, reported in Supplementary Fig. S7.4, showed that co-transfection of miR-194 with PSME3 or CUL4A 3’UTR did not inhibit luciferase activity, meaning that despite the anti-correlated expressions and the presence of a predicted binding site, miR-194 did not directly mediate the inhibition of PSME3 and CUL4A gene expression. On the other hand, when co-transfected with BMI1 3’UTR, miR-194 inhibited luciferase activity by approximately 30% and the difference is significant to the t-test, confirming the results previously reported by Dong et al (14) (Supplementary Fig. S7.4).
DISCUSSION

Since the introduction of platinum compounds in the EOC adjuvant treatment in the 1980s, there has been little improvement in the outcome. One of the possible explanations of the failure of virtually all clinical trials with new drugs in the last decades is that, most of them, as well as most of the EOC studies, did not consider any histotype patients stratifications. Focusing on miRNAs, that are highly tissue-specific and have recently been identified as targets for therapeutic interventions, we investigated the expression and the role of miRNAs in the four EOC histotypes. The results of the analysis indicate an unambiguous miRNA marker set for clear cell (high levels of miR-30a/30a*) and mucinous subtypes (high levels of miR-192/194).

Clear cell subtype markers: miR-30a and miR-30a*

miR-30a/30a* are in log scale five-fold more highly expressed in clear cell subtype than in the other subtypes, as well as in HOSE cells; unfortunately miR-30a* has hitherto not been well characterized. miR-30a negatively regulates BECLIN1, a positive regulator of the autophagy pathway (15), which is a tightly regulated catabolic process considered a key pathway in cancer, even if its role in carcinogenesis has not been fully elucidated (16). Nevertheless, these findings hint tantalizingly at a possible role of autophagy in determining the sensitivity and resistance to clear cell EOC therapy. At time of writing, the potential consequences of these data are not clear.

We found that miR-30a is significantly negatively correlated with E2F3 expression. Specifically, clear cell subtype has significantly lower levels of E2F3 compared to the other subtypes. E2F3 is a transcription factor crucially involved in EOC cell proliferation. It is a prognostic factor positively correlated with the grade of the disease (17,18). Considering all the EOC stages together, Reimer et al, demonstrated that (i) E2F3 levels are highly expressed in EOC compared to normal tissues, (ii) its expression increases with the increase in grade of the disease, and (iii) all the histological subtypes contribute equally to this increment of E2F3 expression. Unfortunately their sample batch lacked of clear cell subtype. In our clear cell samples, we registered a lower E2F3 expression compared to the
other histotypes. Our results suggests that E2F3 underpins a different mechanism and regulation in the clear cell histotype compared to others, perhaps related to miR-30a differential expression. In studies of advanced stages EOC, miR-30a has been already found overexpressed in clear cell histotype (19,20), thus indicating the possibility that miR-30a could be a stage-independent clear cell marker.

**Mucinous subtype markers: miR-192 and miR-194**

miR-192/194 were found to be much more expressed in mucinous than in the other subtypes and in HOSE cells. Borderline mucinous tumors, which are considered putative precursor lesions of malignant mucinous EOCs (2), have approximately the same expression level of miR-192/194 as the malignant mucinous counterpart.

miR-192/194 expression in mucinous samples shows only a slight decrease from borderline to grade 3 (Supplementary Section S8). Accordingly to our findings, low levels of miR-192/194 are known to be associated with a more malignant status in a panel of solid tumors (21–25) especially in colon and gastric tissues in which they are considered biomarkers (22,24). The molecular similarity between mucinous EOC and the colon and gastric carcinomas invites to speculate that mucinous EOC should not be considered and of consequence treated, with the same regimes used for the other EOC histotypes. The integration of gene and miRNA expressions revealed a series of interesting putative targets of miR-192/194, among them CIAO1, PTGER3, BMI1, PSME3 and CUL4A. CIAO1 is a member of the WD40 family of proteins, involved in iron-sulphur protein biogenesis, pertinent to DNA metabolism and genome integrity (26). PTGER3 is a prostanoid receptor, a G-protein-coupled receptor. These genes are involved in multiple genetic diseases, but they have never been associated to EOC. BMI1, PSME3 and CUL4A belong to the p53 regulatory pathway, which differentiates the mucinous from the other ovarian histotypes.

**Differential regulation of pathways upstream p53 in the mucinous EOC**
The expression of miR-192/194 cluster is directly controlled by wild type TP53 that, enhancing their transcription, is able to down-regulate genes of G1-G2 phases, targets of these two miRNAs, arresting cell cycle (26). Among the targets of miR192/194 is MDM2, a negative regulator of TP53 (27). These relationships define a positive feedback loop involving TP53 that, through miR192/194, inhibits its own inhibitor (Fig. 3B). This positive feedback loop TP53-miR192/194-MDM2 confers the status of tumor suppressor to the miR-192/194 cluster. Recently, the importance of this circuit has been further strengthened by the identification of new oncogenes among the down-regulated targets genes of these miRNAs (14,28,29). In our analysis, PSME3 and CUL4A are promising miR-192/194 targets, being among the miR-194 anti-correlated predicted target genes and proteins that physically associate with MDM2 and part of the TP53 degradation pathway. Specifically, PSME3 is a proteasome activator that promotes the nuclear export of TP53 by operating multiple monoubiquitylation, enhancing its physical interaction with MDM2 (30). CUL4A is a Cullin family member that physically associates with MDM2 and participates as a scaffold in the process of polyubiquitylation of TP53 (31) and in its consequent degradation. However, our binding assay reveals that no direct binding occurs between miR-194 and any of these two genes.

Interestingly, another element linked to this pathway is BMI1, a miR-194 anti-correlated target gene, that is a repressor of the CDKN2A protein (32) that prevents the degradation and inactivation of TP53 operated by MDM2 (33). BMI1 belongs to the polycomb group (PcG) of proteins that form chromatin-modifying complexes commonly deregulated in cancer. BMI1 is known to be significantly overexpressed in ovarian, endometrial and cervical cancer compared to normal tissue, and its expression is positively correlated with grade and clinical phases of the disease (34,35). Notably, miR-194 binding on BMI1 mRNA was experimentally validated with the luciferase assay in a panel of endometrial cancer cell lines (14) and re-confirmed by our assay. In our dataset, BMI1 were negatively correlated with expression levels of miR-194, down-regulated in mucinous and up-regulated in other histotypes, confirming the differential activation of the signaling circuit in mucinous compared to other EOC subtypes. Lastly, MDM2 expression and CDKN2A were evaluated using qRT-PCR in all samples.
Mucinous samples showed down-regulated *MDM2* expression and up-regulated *CDKN2A*, consistently with the expression and the relationship among the other elements of the pathway. Taken together our results suggest an opposite regulation of *TP53* circuit in the mucinous subtype, as compared to the other EOC histotypes.

**Conclusions**

Ovarian carcinoma histological subtypes are being recognized as separate disease entities. Nevertheless, current standard treatments are principally guided by the primary origin organ site and not by commonalities in molecular alterations (36). However, some attempt exists, as an example, the on-going study led by the Medical Research Council on advanced stage mucinous EOC (Clinical Trial Identifier: NCT01081262), in which standard paclitaxel/carboplatin chemotherapy is compared with a combination of Oxaliplatin/Capecitabine, a regimen commonly used in colon cancer. Given this scenario, the identification of subtype-specific biomarkers and the understanding of mechanisms that characterize the tumor might allow the development of more efficient strategies aimed at improving the diagnosis and treatments.

In summary, our results demonstrate that *i*) early stage EOC microRNA pattern is different across subtypes, with no overlaps with miRNA signature generated in normal surface epithelium cells; *ii*) mucinous borderline tumors show similar miRNA markers of mucinous tumor of grade 1, 2 and 3, and *iii*) early stage EOC subtypes seem characterized by specific molecular circuits and mechanisms that differentiate the biological features of different histotypes. In this study, we found that miR-30a and miR192/194 are key markers, respectively, of clear cell and mucinous subtypes, that are also ideal candidates for developing new therapeutic strategies, as they show the lowest sensitivity to standard carboplatin-paclitaxel chemotherapy (37). Moreover, considering that the miRNA markers are known to play important roles in other cancer diseases, these results hint tantalizingly the possibility to consider our results as starting point for the study of new EOC treatments. In the light of these findings, we plan to verify our results in sample sets from stage III/IV EOCs, as this could highlight
the difference between early and advanced EOCs and potentially clarify for each single histotype the natural progression of the disease.

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Authors' Contributions

SM, CR and MDI formulated the hypothesis and supervised the study. EC, AR, and RF designed the experiments. RF, SP, ES, MR, DK, LCe and GM collected the samples and clinical data. GC, PP and GT did the histopathological analysis. LP, EB, MDM, LZ and IFN did the experiments. EC, DC, GS and PM did the statistical analysis and developed the computational framework. LCl and LB guaranteed the bioinformatic network and performed the luciferase data analysis. LB submitted data to ArrayExpress. All authors critically reviewed the manuscript and approved the final version.
References


Figure Legends

Figure 1: Flow chart of the experimental and computational strategies of the study.

Figure 2: Microarray Data Analyses. Panel A: heat-map with cluster analysis on miRNA and samples using all the expression profiles. Panel B: bar-plot showing the number of differentially expressed miRNA across subtype comparisons. Panel C: scatterplots and boxplots of the four miRNA marker expression values (miR-194, miR-192, miR-30a, miR-30a*) in patients divided by histotype. Panel D: cluster analysis obtained using only the four subtype-specific miRNAs. Grade and subtypes are reported in different colors.

Figure 3: Differential regulation of pathway upstream p53 in the EOC histotypes. Panel A: Genes and miRNAs qRT-PCR expression values of the circuit in the entire cohort of patients (n=257). Panel B: Schema of the p53 circuit. Red and green in color bar represent high expression and low expression respectively in Endometrioid (E), Clear Cell (C), Mucinous (M), and Serous (S) histotypes. BMI1 is a repressor of CDKN2A protein (32), that prevents the degradation and inactivation of p53 operated by MDM2 (33). PSME3 and CUL4A, interacting with MDM2, promote the TP53 degradation (30,31). Moreover, TP53 is the transcription factor that controls the expression of miR-192/194 cluster (27). Among the targets of this miRNA cluster, miR-194 down regulates BMI1 and both miR-192/194 target MDM2 (14,27). *** means p-value < 1E-07.
Collection A (n=40)

Collection B (n=167)
- B1
- B2
- B3

Collection C (n=50)

Training set

Gene expression (GSE 8841-2)
B1 = 76 patients

microRNA Expression
A + B1 + B2 = 183 patients

Expression Integration

Marker Identification

miRNA markers and putative target qRT-PCR validations
A + B1 + B2 = 183 patients

Validation set

miRNA markers and putative target qRT-PCR validations
B3 + C = 74 patients
Figure 3

A

<table>
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<th>Gene</th>
<th>hsa-miR-192</th>
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<tr>
<td><strong>PSME3</strong></td>
<td></td>
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</tbody>
</table>

Fluorescence intensity normalized (arbitrary units)

---

B

miR-192/194

E C M S

BMI-1

CDKN2A

MDM2

TP53

CUL4A

PSME3

High expression

Low expression
| miRNAs | Class comparison | With Borderlines | | | | Without Borderlines | | | |
|---|---|---|---|---|---|---|---|---|
| miR-192 | Muc vs. Cc | log₂(FC) | adj.P.Val | RS | log₂(FC) | adj.P.Val | RS |
| | | 4.58 | 2.36E-19 | 500 | 4.09 | 1.40E-13 | 500 |
| | Muc vs. End | 4.46 | 1.80E-21 | 500 | 4.01 | 2.03E-14 | 500 |
| | Muc vs. Ser | 4.29 | 1.93E-20 | 500 | 3.6 | 3.81E-11 | 500 |
| miR-194 | Muc vs. Cc | 4.49 | 1.27E-16 | 500 | 3.83 | 1.96E-11 | 500 |
| | Muc vs. End | 4.01 | 6.09E-17 | 500 | 3.39 | 1.65E-10 | 500 |
| | Muc vs. Ser | 4.15 | 2.75E-18 | 500 | 3.39 | 9.37E-10 | 500 |
| miR-30a | Cc vs. End | 2.18 | 6.94E-13 | 500 | 2.15 | 2.67E-11 | 500 |
| | Cc vs. Muc | 2.47 | 1.27E-16 | 500 | 2.63 | 5.37E-13 | 500 |
| | Cc vs. Ser | 1.99 | 8.08E-11 | 500 | 2.08 | 1.50E-09 | 500 |
| miR-30a | Cc vs. End | 1.62 | 4.67E-07 | 494 | 1.62 | 6.96E-06 | 475 |
| | Cc vs. Muc | 1.91 | 8.94E-10 | 500 | 2.00 | 3.14E-06 | 487 |
| | Cc vs. Ser | 1.28 | 8.72E-05 | 408 | 1.39 | 0.000424 | 316 |

**Table I:** Microarray measurements of miRNA markers across histotypes. For each comparison, data including and excluding borderline tumors are shown. Mucinous (Muc), clear cell (Cc), serous (Ser), endometrioid (End); fold change is in log₂ scale (log₂(FC)), P-value were adjusted for multiple testing (adj.P.Val), resampling score (RS).
### Table II: qRT-PCR measurements of miRNA markers across histotypes. For each comparison, data including and excluding borderline tumors are shown. Mucinous (Muc), clear cell (Cc), serous (Ser), endometrioid (End); fold change is in log₂ scale (log₂(FC)).

<table>
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<tr>
<th>miRNAs</th>
<th>Class comparison</th>
<th>Training set</th>
<th>Validation set</th>
<th>Training set</th>
<th>Validation set</th>
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<tr>
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<td>log₂(FC)</td>
<td>p-value</td>
<td>log₂(FC)</td>
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<td>8.79</td>
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<td>5.73</td>
<td>7.33E-06</td>
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<td><strong>Clear cell</strong></td>
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<tr>
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<td>1.37E-11</td>
<td>6.54</td>
<td>2.68E-05</td>
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<tr>
<td></td>
<td>Cc vs. Ser</td>
<td>7.19</td>
<td>1.96E-15</td>
<td>6.63</td>
<td>2.72E-06</td>
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miRNA landscape in Stage I Epithelial Ovarian Cancer defines the histotype specificities

Enrica Calura, Robert Fruscio, Lara Paracchini, et al.

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